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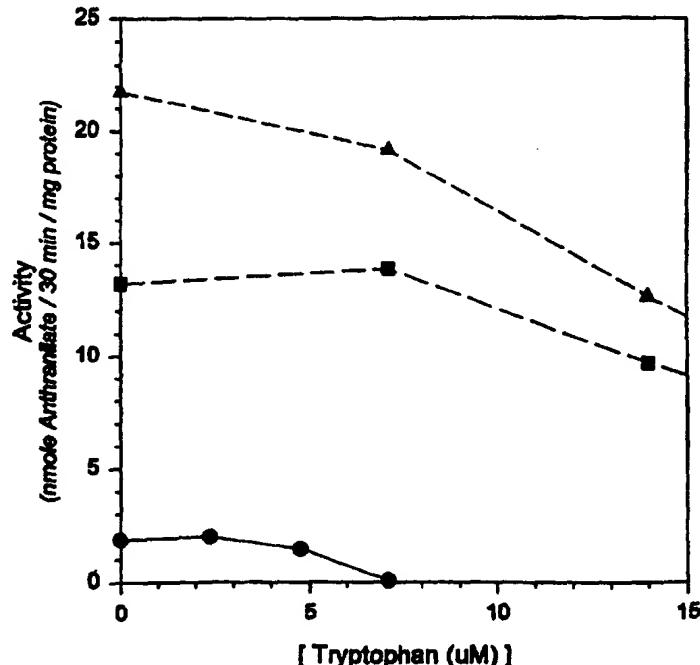
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| (71) Applicant (for all designated States except US): DEKALB GENETICS CORPORATION [US/US]; 3100 Sycamore Road, DeKalb, IL 60115 (US). | | | |
| (72) Inventors; and | | | |
| (75) Inventors/Applicants (for US only): ANDERSON, Paul, C. [US/US]; 849 - 25th Street, West Des Moines, IA 50265 (US). CHOMET, Paul, S. [US/US]; 204 Evarts Lane, Mystic, CT 06355 (US). GRIFFOR, Matthew, C. [US/US]; 386 Wyassup Road, North Stonington, CT 06359 (US). KRIZ, Alan, L. [US/US]; 23 Robin Hood Drive, Gales Ferry, CT 06335 (US). | | | |
| (74) Agent: VIKSNINS, Ann, S.; Schwegman, Lundberg, Woessner & Kluth, P.O. Box 2938, Minneapolis, MN 55402 (US). | | | |

(54) Title: ANTHRANILATE SYNTHASE GENE AND USE THEREOF

(57) Abstract

The present invention provides a method for conferring tolerance to an amino acid analog of tryptophan to a plant and/or altering the tryptophan content of a plant by introducing and expressing an isolated DNA segment encoding an anthranilate synthase in the cells of the plant. Transgenic plants transformed with an isolated DNA segment encoding an anthranilate synthase, as well as seeds and progeny derived from these plants, are also provided. The present invention also provides a cDNA sequence of an alpha and a beta subunit of a maize anthranilate synthase.

| | |
|---|----------------------|
| ● | TRPA013 - control |
| ■ | TRPA015 - medium trp |
| ▲ | TRPA030 - high trp |



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ANTHRANILATE SYNTHASE GENE AND USE THEREOF

5

Background of the Invention

Anthranilate synthase (AS) catalyzes the first reaction branching from the aromatic amino acid pathway to the biosynthesis of tryptophan in plants, fungi, and bacteria. The reaction catalyzed by anthranilate synthase is the conversion of chorismate to anthranilate in a glutamine-dependent reaction. In 10 microorganisms, anthranilate synthase is composed of two non-identical subunits: the alpha subunit binds chorismate and eliminates the enolpyruvate side chain, and the beta subunit transfers an amino group from glutamine to the position vacated by the enolpyruvate moiety.

The next reaction in the synthesis of tryptophan is the transfer of 15 the phosphoribosyl moiety of phosphoribosyl pyrophosphate to anthranilate. The indole ring is formed in two steps involving an isomerization converting the ribose group to a ribulose followed by a cyclization reaction to yield indole glycerol phosphate. The final reaction in the pathway is catalyzed by a single enzyme that may contain either one or two subunits. The reaction accomplishes 20 the cleavage of indole glyceraldehyde-3-phosphate and condensation of the indole group with serine (Umbarger, *Ann. Rev. Biochem.*, **47**, 555 (1978)).

Metabolite flow in the tryptophan pathway in higher plants and 25 microorganisms is apparently regulated through feedback inhibition of anthranilate synthase by tryptophan. Thus, because anthranilate synthase is feedback inhibited by tryptophan, the overproduction of wild-type anthranilate synthase cannot result in tryptophan overproduction.

While anthranilate synthase has been partially purified from crude extracts of cell cultures of higher plants (Hankins et al., *Plant Physiol.*, **57**, 101 (1976); Widholm, *Biochim. Biophys. Acta*, **320**, 217 (1973)), it was found to be 30 very unstable. In order to further characterize the anthranilate synthase of plants, Niyogi and Fink (*Plant Cell*, **4**, 721 (1992)) and Niyogi et al. (*Plant Cell*, **5**, 1011 (1993)) employed a molecular approach. They found that *Arabidopsis* anthranilate synthase alpha subunits are encoded by two closely related, nonallelic genes which are differentially regulated. One of these alpha subunit

genes, ASA1, is induced by wounding and bacterial pathogen infiltration, implicating its involvement in a defense response, whereas the other alpha subunit gene, ASA2, is expressed at constitutive basal levels. Both predicted proteins share regions of homology with bacterial and fungal anthranilate synthase proteins, and contain conserved amino acid residues at positions that have been shown to be involved in tryptophan feedback inhibition in bacteria (Caligiuri et al., *J. Biol. Chem.*, **266**, 8328 (1991)).

Amino acid analogs of tryptophan or of intermediates in the tryptophan biosynthetic pathway (e.g., 5-methyltryptophan, 4-methyltryptophan, 5-fluorotryptophan, 5-hydroxytryptophan, 7-azatryptophan, 3 β -indoleacrylic acid, 3-methylantranilic acid) have been shown to inhibit the growth of both prokaryotic and eukaryotic cultures. Plant cell cultures can be selected for resistance to these amino acid analogs. For example, cultured tobacco, carrot, potato, corn and *Datura innoxia* cell lines have been selected which are resistant to growth inhibition by 5-methyltryptophan (5-MT), an amino acid analog of tryptophan, due to expression of an altered anthranilate synthase as described below.

Widholm (*Biochem. Biophys. Acta*, **261**, 44 (1972)) demonstrated that the tryptophan analogs 5-MT, 4-methyltryptophan, 5-fluorotryptophan and 6-fluorotryptophan cause growth inhibition of tobacco (*Nicotiana tabacum*) and carrot (*Daucus carota*) cell cultures. This inhibition of growth could be reversed by the addition of anthranilic acid, indole, or L-tryptophan. Anthranilate synthase was determined to be very sensitive to these analogs. The tryptophan analogs inhibited cell growth by limiting tryptophan synthesis through the inhibition of anthranilate synthase.

While growth of many cultured tobacco cell lines was inhibited by 5-MT, some tobacco cell lines were resistant to growth inhibitory concentrations of 5-MT (Widholm, *Biochim. Biophys. Acta*, **261**, 52 (1972)). The resistant phenotype was stable for at least 60 cell mass doublings even without selection pressure (i.e., without 5-MT). In addition, 5-MT resistant cells were resistant to growth inhibition by other tryptophan analogs. Free tryptophan

levels were increased in 5-MT resistant cells about 10-fold over control tissue. Anthranilate synthase in these 5-MT resistant cells was found to be less sensitive to inhibition by tryptophan or 5-MT.

Carrot cell lines that were resistant to growth inhibition by 5-MT
5 were also selected by Widholm (Biochim. Biophys. Acta, 279, 48 (1972)). This phenotype was generally stable in the absence of the tryptophan analog for at least 100 cell doublings. 5-MT resistant cells were also resistant to other tryptophan analogs. Free tryptophan concentrations in 5-MT resistant cells were substantially increased to 2170 μ M as compared to 81 μ M (27-fold) for control
10 tissue. Anthranilate synthase was shown to be altered in the 5-MT resistant cells. The enzyme was about 5-fold less sensitive to inhibition by tryptophan or 5-MT than an unaltered anthranilate synthase.

Singh et al. (Biochem. Genet., 13, 357 (1975)) described a mutant in corn, *Zea mays* L., *blue fluorescent-1*, that possessed increased anthranilate
15 synthase activity which was less sensitive to feedback inhibition. The mutant also accumulated anthranilic acid. In contrast to previous work in tobacco and carrot, however, the altered anthranilate synthase activity did not lead to significant overproduction of tryptophan in mature corn plants or seed.

Hibberd et al. (U.S. Patent No. 4,581,847, issued April 15, 1986)
20 described 5-MT resistant maize cell lines that contained an anthranilate synthase that was less sensitive to feedback inhibition than wild-type anthranilate synthase. One 5-MT resistant cell line accumulated free tryptophan at levels almost twenty-fold greater than that of non-transformed cell lines.

Carlson et al. (Physiol. Plant., 44, 251 (1978)) obtained potato cell
25 (*Solanum tuberosum*) cultures resistant to 5-MT. Anthranilate synthase in these cultures was shown to be less sensitive to inhibition by tryptophan or by 5-MT, although both 5-MT resistant and sensitive forms of the enzyme were present in the cells of the culture. In the selected cell lines, the level of resistant anthranilate synthase was greatly increased relative to the level of the sensitive
30 form. The range of free tryptophan concentrations in selected cultures was from

970 to 1400 μ M compared to control cultures in which the tryptophan concentrations were about 29 μ M.

Widholm (Plant Cell Cultures: Results and Perspectives, F. Sala,

B. Parisi, R. Cella, O. Ciferri (eds.), Elsevier/North Holland Biomedical Press,

5 Amsterdam, pp. 157-159 (1980)) described plants regenerated from 5-MT resistant *N. tabacum* suspension cultures. While the cultures expressed an anthranilate synthase enzyme that was less sensitive to feedback inhibition by tryptophan and also exhibited an increased level of free tryptophan (approximately 25-fold), the leaves of the regenerated plants did not express the 10 altered form of the enzyme and did not form roots in medium containing 5-MT. The resistance trait was, however, expressed in callus derived from the regenerated plant. Thus it appears to be difficult to obtain expression of the 5-MT resistance phenotype in tobacco plants derived from 5-MT resistant cells selected in culture.

15 Finally, Ranch et al. (Plant Physiol., 71, 136 (1983)) selected for 5-MT resistance in cell cultures of *Datura innoxia*, a dicot weed, and reported that the resistant cell cultures contained increased tryptophan levels (8 to 30 times higher than the wild type level) and an anthranilate synthase with less sensitivity to tryptophan feedback inhibition. Regenerated plants were also 20 resistant to 5-MT, contained an altered anthranilate synthase, and had a greater concentration of free tryptophan (4 to 44 times) in the leaves than in the leaves of the control plants. In contrast to the studies with *N. tabacum*, where the altered enzyme was not expressed in plants regenerated from resistant cell lines, these results indicated that the amino acid overproduction phenotype could be selected 25 at the cellular level and expressed in whole plants regenerated from the selected cells in *Datura innoxia*.

Although it is possible to select for 5-MT resistance in certain cell cultures and plants, this characteristic does not necessarily correlate with the overproduction of free tryptophan in whole plants. Additionally, plants

30 regenerated from 5-MT resistant lines frequently do not express an altered form

of the enzyme. Nor is it predictable that this characteristic will be stable over a period of time and will be passed along as a heritable trait.

Thus, there is a need to increase the tryptophan content of plants and/or provide plants that are resistant to growth inhibitory amounts of tryptophan or an analog thereof.

Summary of the Invention

The present invention provides an isolated and purified DNA molecule comprising a DNA segment encoding an anthranilate synthase (AS) substantially resistant to inhibition by free L-tryptophan or an amino acid analog of tryptophan such as those discussed hereinbelow. A preferred embodiment of the invention is a plant anthranilate synthase which is substantially resistant to inhibition by free L-tryptophan or an amino acid analog of tryptophan. Another embodiment of the invention is an isolated and purified DNA molecule comprising a DNA segment encoding a maize anthranilate synthase. A DNA molecule of the invention can further comprise an amino terminal plant chloroplast transit peptide sequence operably linked to the anthranilate synthase coding sequence.

Another embodiment of the invention provides a method for conferring tolerance to an amino acid analog of tryptophan and/or altering the tryptophan content of a plant, plant tissue, plant part, or plant cell by introducing and expressing a DNA segment encoding an anthranilate synthase in a plant cell. Also provided is a method of molecularly detecting an anthranilate synthase that is substantially tolerant to inhibition by an amino acid analog of tryptophan comprising identification of a restriction enzyme site polymorphism in the gene encoding the anthranilate synthase. The method comprises mixing DNA isolated from a culture, organism, or a portion of an organism, with an amount of NlaIII restriction endonuclease, and determining whether the restriction endonuclease cleaves the DNA within a portion of the DNA that encodes an anthranilate synthase.

The method of imparting tolerance to an amino acid analog of tryptophan to a plant, plant cell, plant part or plant tissue includes the steps of introducing a preselected DNA segment encoding an anthranilate synthase that is substantially tolerant to inhibition by the tryptophan analog or free L-tryptophan

5 and that is operably linked to a promoter functional in a plant cell, into cells of a susceptible plant. The transformed plant cells are then regenerated to provide a differentiated fertile plant. The promoter can be an inducible or tissue specific promoter. The functional linkage of a promoter to the DNA segment results in an expression cassette. Other transcription or translation regulatory elements,

10 e.g., enhancers or terminators, can also be functionally linked to the DNA segment. Expression of the DNA segment yields an amount of anthranilate synthase effective to render the plant, plant part, plant cell, or plant tissue substantially tolerant to an amount of an amino acid analog of tryptophan or free L-tryptophan that inhibits the growth of a corresponding plant, plant part, plant

15 cell or plant tissue without the DNA segment ("untransformed" material).

Once transformed plant cells exhibiting tolerance are obtained, transgenic plants can then be regenerated therefrom, and evaluated for stability of the inheritance of the resistance or tolerance trait, that is, whether the resistance or tolerance trait is transmitted to progeny. Thus, another embodiment of the invention is a transformed plant that is substantially tolerant to an amino acid analog of tryptophan. The cells of the transformed plant comprise a native anthranilate synthase gene and a DNA segment encoding an exogenous anthranilate synthase. The expression of the exogenous anthranilate synthase in the cells of the plant confer tolerance to the plant to an amount of an amino acid analog of tryptophan or free L-tryptophan that inhibits the activity of the native anthranilate synthase.

The transmission of the resistance or tolerance trait can be evaluated at a molecular level, e.g., Southern or Northern blot analysis, PCR-based methodologies, or the biochemical or immunological detection of

30 anthranilate synthase, or by phenotypic analyses, i.e., whether transformed progeny can grow in the presence of an amount of an amino acid analog of

tryptophan or free L-tryptophan that inhibits the growth of an untransformed plant. Also provided is a transformed plant which is substantially tolerant to growth inhibition by an amino acid analog of tryptophan or free L-tryptophan, as well as a seed therefrom.

5 The invention also provides a method for altering, preferably increasing, the tryptophan content in a plant. The method comprises introducing a DNA segment comprising a gene encoding an anthranilate synthase substantially resistant to inhibition by free L-tryptophan or an amino acid analog of tryptophan, wherein the segment is operably linked to a promoter functional
10 in a plant cell, into the cells of a plant and then expressing the gene in an amount effective to alter the tryptophan content of the plant cell. Thus, another embodiment of the invention is a transformed plant having an altered cellular tryptophan content.

In a preferred embodiment of the invention, transformed cells
15 exhibiting about a 1.1- to 50-fold increase in total tryptophan content are selected for and used to generate transgenic plants, plant parts and seeds exhibiting a substantial increase in tryptophan content. A substantial increase in tryptophan content is determined with respect to the tryptophan content normally present in the untransformed plant, plant part, e.g., leaves or fruit, or seed, and
20 can range from about a 1.1 to a 50-fold increase over that present in the corresponding untransformed plant.

Also provided is a fertile transgenic *Zea mays* plant comprising a DNA segment encoding an anthranilate synthase that is substantially resistant to inhibition by free L-tryptophan or an amino acid analog of tryptophan, operably linked to a promoter. The expression of this DNA segment in the transgenic *Zea mays* plant results in levels of free L-tryptophan in the cells of the transgenic plant that are substantially increased above the levels of free L-tryptophan in the cells of a *Zea mays* plant which only differ from the cells of the transgenic *Zea mays* plant by the absence of the DNA segment. This DNA segment is
25 transmitted through a complete normal sexual cycle of the plant to its progeny and to further generations.

The invention also provides for a method of producing anthranilate synthase in a host cell. The method includes the steps of introducing an expression cassette comprising a DNA segment encoding an anthranilate synthase into a host cell and expressing the DNA segment in the host cell so as

5 to yield anthranilate synthase. An expression cassette preferably includes transcription and translation regulatory elements, e.g., a promoter, functional in host cell, either of eukaryotic or prokaryotic origin. Preferably, the expression cassette is introduced into a prokaryotic cell, such as *E. coli*, or a eukaryotic cell, such as a yeast or insect cell, that is known to be useful for production of

10 recombinant proteins. Recombinantly produced anthranilate synthase can then be used to identify other agents that bind to and inhibit anthranilate synthase.

The present invention also provides an isolated and purified DNA molecule of at least seven nucleotide bases which hybridizes under high stringency conditions to a DNA molecule comprising a DNA segment encoding

15 an anthranilate synthase substantially resistant to inhibition by free L-tryptophan or an amino acid analog of tryptophan, such as a plant anthranilate synthase. Also provided is a hybridization probe comprising an isolated and purified DNA segment of at least seven nucleotide bases, which is detectably labeled or which can bind to a detectable label, which DNA segment hybridizes under high

20 stringency conditions to the non-coding strand of a DNA molecule comprising a DNA segment encoding an anthranilate synthase, such as a plant anthranilate synthase, substantially resistant to inhibition by free L-tryptophan or an amino acid analog of tryptophan, or a maize anthranilate synthase. High stringency conditions are defined as: hybridization at 65°C for at least 16 hours in 5xSSC,

25 1x Denhardt's solution, 50 mM Tris-HCl, pH 8, 0.2% SDS, 10 mM EDTA, 0.1 mg/ml salmon sperm DNA, followed by washing twice for 5 minutes in 2xSSC, 0.5% SDS at 25°C, once for 10 minutes in 0.2xSSC, 0.1% SDS at 25°C and twice for 30 minutes in 0.2xSSC, 0.1% SDS at 56°C.

The present invention also provides a method of introducing an

30 exogenous anthranilate synthase gene into a host cell comprising transforming host cells *in vitro* with an expression cassette comprising a DNA segment

encoding an anthranilate synthase, operably linked to a promoter functional in the host cell, expanding the transformed host cells *in vitro*, and identifying a transformed host cell which expresses the anthranilate synthase encoded by the DNA segment. A preferred embodiment of the invention is method of

5 introducing an exogenous anthranilate synthase gene into plant cells. A more preferred embodiment of the invention is a method wherein the transformed plant cells can be regenerated into a differentiated dicot or monocot plant.

Another embodiment of the invention is a method of selecting transformed plant cells. The method comprises introducing a preselected DNA

10 segment into a plant cell to yield a transformed plant cell. The DNA segment encodes an anthranilate synthase which is substantially resistant to inhibition by free L-tryptophan or an amino acid analog of tryptophan. The transformed cell is cultured in an amount of free L-tryptophan or an amino acid analog of tryptophan that inhibits the growth of a plant cell which does not contain the

15 preselected DNA segment. A preferred embodiment of the invention is a DNA segment encoding a plant anthranilate synthase. Another preferred embodiment of the invention is a method of selecting transformed plant cells comprising introducing a preselected DNA segment into a plant cell to yield a transformed plant cell. The DNA segment encodes a chloroplast transit peptide operably

20 linked to an anthranilate synthase which is substantially resistant to inhibition by free L-tryptophan or an amino acid analog of tryptophan.

Also provided is an isolated and purified DNA molecule comprising a DNA segment encoding transcription regulatory elements for a plant anthranilate synthase gene.

25 Further provided is an isolated and purified DNA molecule comprising a DNA segment encoding a beta subunit of an anthranilate synthase.

The term "consists essentially of" as used with respect to the present DNA molecules, sequences or segments is defined to mean that a major portion of the nucleotide sequence encodes an anthranilate synthase, optionally

30 operably linked to a chloroplast transit peptide, and that nucleotide sequences are

not present which encode proteins other than an anthranilate synthase, optionally operably linked to a chloroplast transit peptide.

As used herein, "an amino acid analog of tryptophan" is an amino acid analog of an intermediate in the tryptophan biosynthetic pathway or an 5 amino acid analog of tryptophan. These analogs include, but are not limited to, 6-methylanthranilate, 5-methyltryptophan, 4-methyltryptophan, 5-fluorotryptophan, 5-hydroxytryptophan, 7-azatryptophan, 3 β -indoleacrylic acid, 3-methylanthranilic acid, and the like.

As used herein, "substantially increased" or "elevated" levels of 10 free L-tryptophan in a plant cell, plant tissue, or plant are levels that are about 1.1 to 50 times, preferably about 2 to 20 times, and more preferably about 3-10 times, the levels found in an untransformed plant cell, plant tissue, or plant, i.e., one where the genome has not been altered by the presence of an exogenous gene. For example, the levels of free L-tryptophan in a transformed plant are 15 compared with those in an untransformed plant. In the alternative, the levels of free L-tryptophan in a homozygous backcross converted inbred transformed plant are compared to the levels in a recurrent inbred plant. A homozygous backcross converted inbred transformed plant is a transformed plant which has been repeatedly crossed to the recurrent inbred parent until the transformed plant 20 is substantially isogenic with the recurrent inbred parent except for the presence of an introduced transgene, and is then is self-pollinated (selfed) at least once.

As used herein, "substantially isogenic" means that the genomic DNA content of a homozygous backcross converted inbred transformed plant is at least about 92%, preferably at least about 98%, and most preferably at least 25 about 99%, identical to the genomic DNA content of a recurrent inbred parent of the transformed plant.

As used herein, a plant cell, plant tissue or plant that is "substantially resistant or tolerant to inhibition by free L-tryptophan or an amino acid analog of tryptophan" is a plant cell, plant tissue; or plant that grows in an 30 amount of tryptophan or an amino acid analog of tryptophan that normally inhibits growth of the untransformed plant cell, plant tissue, or plant, as

determined by methodologies known to the art. For example, a homozygous backcross converted inbred plant transformed with a DNA molecule that encodes an anthranilate synthase that is substantially resistant or tolerant to inhibition by free L-tryptophan or an amino acid analog of tryptophan grows in an amount of 5 tryptophan or an amino acid analog of tryptophan that inhibits the growth of the corresponding, i.e., substantially isogenic, recurrent inbred plant.

As used herein, an anthranilate synthase that is "substantially resistant or tolerant to inhibition by free L-tryptophan or an amino acid analog of tryptophan" is an anthranilate synthase that is not inhibited by an amount of free 10 L-tryptophan or an amino acid analog of tryptophan that normally inhibits the corresponding "wild-type" or native anthranilate synthase of the species.

As used herein, "cells of a susceptible plant" are cells which are sensitive to growth inhibition by free L-tryptophan or to an amino acid analog of tryptophan. For example, plant cells from the C28 maize cell line are not 15 susceptible to growth inhibition by 5-methyltryptophan at levels of 5-methyltryptophan that inhibit the growth of many other maize cell lines, such as those obtained from the inbred line H99 or from hybrids such as A188 x B73 (Miao et al., Plant Cell, Tissue and Organ Culture, 14, 3 (1988)). Thus, C28 cells are not cells of a susceptible plant.

20 As used herein, an "exogenous" anthranilate synthase is an anthranilate synthase that is encoded by a DNA sequence that has been isolated from a cell, purified, and amplified.

As used herein, a "native" gene means a gene that has not been manipulated *in vitro*, i.e., has not been isolated, purified, and amplified.

25 As used herein, "altered" levels of tryptophan in a transformed plant, plant tissue, or plant cell are levels which are greater than the levels found in the corresponding untransformed plant, plant tissue, or plant cells. In the alternative, altered levels of tryptophan in a backcross converted inbred transformed plant are greater than the levels found in the corresponding recurrent 30 inbred plant.

Brief Description of the Figures

Figure 1 is a schematic diagram of clone pASA2 2-1.

Figure 2 is the nucleotide sequence of the maize anthranilate synthase alpha subunit gene (SEQ ID NO:1).

5 Figure 3 is an alignment of the predicted amino acid sequence of maize ASA2 2-1 (SEQ ID NO:2) with those of the *Arabidopsis* anthranilate synthase ASA1 alpha subunit (SEQ ID NO:3) and the *Arabidopsis* anthranilate synthase ASA2 alpha subunit (SEQ ID NO:4). Dots indicate residues implicated in feedback regulation in the *Salmonella* *TrpE* gene (Caligiuri and Bauerle, *J. Biol. Chem.* **266**, 8328 (1991)). Arrows indicate position of M-K change in C28 allele.

10 Figure 4 is a graph of the activity (nmole anthranilate/30 minutes/mg protein) versus tryptophan concentration (μ M) for 3 transformants (TRPA015, TRPA030, and TRPA013).

15 Figure 5 is the nucleotide sequence of the region immediately upstream of the maize anthranilate synthase alpha subunit coding region (SEQ ID NO:14). The putative ATG for the anthranilate synthase is approximately 130 nucleotides 3' to the 3' most nucleotide in SEQ ID NO:14.

20 Figure 6 is the partial nucleotide sequence of the maize anthranilate synthase beta subunit gene (SEQ ID NO:8). The underlined nucleotides are homologous to nucleotides present in the *Arabidopsis* anthranilate synthase ASB1 beta subunit.

Figure 7 is the predicted amino acid sequence of the C28 allele of maize anthranilate synthase (SEQ ID NO:16).

25

Detailed Description of the Invention

The present invention provides a DNA molecule encoding an anthranilate synthase substantially resistant to inhibition by free L-tryptophan or an amino acid analog of tryptophan. Also provided are methods for conferring 30 tolerance to an amino acid analog of tryptophan and/or altering the tryptophan content of plant tissues and cells by introducing and expressing an anthranilate

synthase gene in the plant cells. Methods and compositions are provided for producing callus cultures, plant tissues, plants and seeds that are tolerant and/or resistant to levels of an amino acid analog of tryptophan or free L-tryptophan that normally inhibit growth. Such plants and seeds genetically can transmit this 5 trait to their progeny.

Methods and compositions are also provided for cloning genes associated with resistance to tryptophan feedback inhibition, and producing callus cultures, plant tissues, plants, plant parts and seeds which overproduce tryptophan and sexually transmit this trait to their progeny. Also described are 10 cell culture selection techniques to select for novel genotypes resistant to tryptophan analogs and which also overproduce tryptophan. For example, to produce resistant maize lines, maize cells that are resistant to a tryptophan analog or free L-tryptophan are isolated and characterized, then regenerated into plants which are resistant to growth inhibition by the analog or by free L-tryptophan. 15 The methods provided in the present invention may also be used to produce increased levels of free tryptophan in monocots and other cereal crops including, but not limited to, rice, rye, millet, wheat, barley, sorghum, and oats.

In accord with the present invention, an anthranilate synthase gene that encodes an enzyme that is resistant to inhibition by free L-tryptophan 20 or an amino acid analog of tryptophan, is identified, isolated, and combined with at least a promoter functional in a plant cell to provide a recombinant expression cassette.

The construction of such expression cassettes which may be employed in conjunction with the present invention will be known to those of 25 skill in the art in light of the present disclosure (see, e.g., Sambrook et al., Molecular Cloning: A Laboratory Manual, Cold Spring Harbor, New York (1989); Gelvin et al., Plant Molecular Biology Manual, (1990)). Preferred constructs will generally include, but are not limited to, a plant promoter such as the CaMV 35S promoter (Odell et al., Nature, 313, 810 (1985)), or others such 30 as CaMV 19S (Lawton et al., Plant Mol. Biol., 2, 31F (1987)), nos (Ebert et al., PNAS USA, 84, 5745 (1987)), Adh (Walker et al., PNAS USA, 84, 6624

(1987)), sucrose synthase (Yang et al., PNAS USA, 87, 4144 (1990)), α -tubulin, actin (Wang et al., Mol. Cell. Biol., 12, 3399 (1992)), *cab* (Sullivan et al., Mol. Gen. Genet., 215, 431 (1989)), PEPCase (Hudspeth et al., Plant Mol. Biol., 12, 579 (1989)) or those associated with the *R* gene complex (Chandler et al., The Plant Cell, 1, 1175 (1989)). It is contemplated that other promoters useful in the practice of the invention are known to those of skill in the art.

Moreover, transcription enhancers or duplications of enhancers can be used to increase expression from a particular promoter. Examples of such enhancers include, but are not limited to, elements from the CaMV 35S promoter 10 and octopine synthase genes (Last et al., U.S. Patent No. 5,290,924, issued March 1, 1994). For example, it is contemplated that vectors for use in accordance with the present invention may be constructed to include the *ocs* enhancer element. This element was first identified as a 16 bp palindromic enhancer from the octopine synthase (*ocs*) gene of *Agrobacterium* (Ellis et al., 15 EMBO J., 6, 3203 (1987)), and is present in at least 10 other promoters (Bouchez et al., EMBO J., 8, 4197 (1989)). It is proposed that the use of an enhancer element, such as the *ocs* element and particularly multiple copies of the element, will act to increase the level of transcription from adjacent promoters when applied in the context of monocot transformation. Tissue-specific promoters, 20 including but not limited to, root-cell promoters (Conkling et al., Plant Physiol., 93, 1203 (1990)), and tissue-specific enhancers (Fromm et al., The Plant Cell, 1, 977 (1989)) are also contemplated to be particularly useful, as are inducible promoters such as ABA- and turgor-inducible promoters, and the like.

As the DNA sequence between the transcription initiation site and 25 the start of the coding sequence, i.e., the untranslated leader sequence, can influence gene expression, one may also wish to employ a particular leader sequence. Preferred leader sequences are contemplated to include those which include sequences predicted to direct optimum expression of the attached gene, i.e., to include a preferred consensus leader sequence which may increase or 30 maintain mRNA stability and prevent inappropriate initiation of translation (Joshi, Nucl. Acid Res., 15, 6643 (1987)). The choice of such sequences will be

known to those of skill in the art. Sequences that are derived from genes that are highly expressed in plants, and in maize in particular, will be most preferred.

Constructs will also include the gene of interest along with a 3' end DNA sequence that acts as a signal to terminate transcription and allow for the polyadenylation of the resultant mRNA. The most preferred 3' elements are contemplated to be those from the nopaline synthase gene of *Agrobacterium tumefaciens* (Bevan et al., Nucl. Acid Res., 11, 369 (1983)), the terminator for the T7 transcript from the octopine synthase gene of *Agrobacterium tumefaciens*, and the 3' end of the protease inhibitor I or II genes from potato or tomato, although other 3' elements known to those of skill in the art are also contemplated. Regulatory elements such as Adh intron 1 (Callis et al., Genes Develop., 1, 1183 (1987)), sucrose synthase intron (Vasil et al., Plant Physiol., 91, 5175 (1989)) or TMV omega element (Gallie et al., The Plant Cell, 1, 301 (1989)) may further be included where desired. Other such regulatory elements useful in the practice of the invention are known to those of skill in the art.

Additionally, expression cassettes may be constructed and employed to provide targeting of the gene product to an intracellular compartment within plant cells or in directing a protein to the extracellular environment. This will generally be achieved by joining a DNA sequence encoding a transit or signal peptide sequence to the coding sequence of a particular gene. The resultant transit, or signal, peptide will transport the protein to a particular intracellular, or extracellular destination, respectively, and will then be post-translationally removed. Transit or signal peptides act by facilitating the transport of proteins through intracellular membranes, e.g., vacuole, vesicle, plastid and mitochondrial membranes, whereas signal peptides direct proteins through the extracellular membrane. By facilitating transport of the protein into compartments inside or outside the cell, these sequences may increase the accumulation of gene product.

A particular example of such a use concerns the direction of an anthranilate synthase to a particular organelle, such as the chloroplast rather than

to the cytoplasm. This is exemplified by the use of the *rbcS* (RuBISCO) transit peptide which confers plastid-specific targeting of proteins.

It is contemplated that targeting of the gene product to an intracellular compartment within plant cells may be achieved by direct delivery

5 of a gene to the intracellular compartment. For example, a gene expression cassette encoding a protein the presence of which is desired in the chloroplast, may be directly introduced into the chloroplast genome using the method described in Maliga et al., U.S. Patent No. 5,451,513, issued September 19, 1995, incorporated herein by reference.

10 It is also contemplated that it may be useful to target DNA itself within a cell. For example, it may be useful to target introduced DNA to the nucleus as this may increase the frequency of transformation. Within the nucleus itself, it would be useful to target a gene in order to achieve site-specific integration. For example, it would be useful to have a gene introduced through

15 transformation replace an existing gene in the cell.

After constructing an expression cassette containing an anthranilate synthase gene, the cassette can then be introduced into a plant cell. Depending on the type of plant cell, the level of gene expression, and the activity of the enzyme encoded by the gene, introduction of an anthranilate synthase gene

20 into the plant cell can confer tolerance to an amino acid analog of tryptophan, such as 5-methyltryptophan or 6-methylanthranilate, and/or alter the tryptophan content of the plant cell.

Also in accord with the present invention, an anthranilate synthase gene, such as a plant anthranilate synthase gene, that encodes an

25 enzyme that is sensitive to inhibition by free L-tryptophan or an amino acid analog of tryptophan, is identified and isolated. Such a gene can be used to screen recombinant cDNA or genomic libraries derived from cells that are resistant to inhibition by free L-tryptophan or an amino acid analog of tryptophan to isolate anthranilate synthase mutants or to introduce specific

30 mutations into the anthranilate synthase coding region that result in an enzyme that is resistant to inhibition by free L-tryptophan or an amino acid analog of

tryptophan. Further, the gene can be combined with a promoter functional in a host cell to provide a recombinant expression cassette. The expression of the gene in the host cell provides amounts of recombinant anthranilate synthase useful for *in vitro* screening of agents for their efficacy of enzyme inhibition.

5 In monocots, an exogenously introduced anthranilate synthase gene that encodes an enzyme that is resistant to inhibition by free L-tryptophan or an analog of tryptophan can be expressed at a level effective to render the cells of the plant tissue substantially tolerant to feedback inhibition by free L-tryptophan at an amount of L-tryptophan that normally inhibits a native or
10 endogenous anthranilate synthase. The tissue can also be rendered resistant to growth inhibition by an amino acid analog of tryptophan at an amount of the analog that normally inhibits the growth of a plant cell or tissue or the activity of a native or endogenous anthranilate synthase. A native or endogenous anthranilate synthase is an enzyme that is normally encoded and expressed in the
15 native plant cell prior to transformation. An exogenously introduced anthranilate synthase gene is a gene which has been isolated from a cell and amplified. Exogenous introduction and expression of an anthranilate synthase gene in both monocots and dicots can result in alteration of the tryptophan content and quality of plant tissue and seeds. Exogenous introduction and expression in a host cell,
20 such as a bacteria, can provide a source for isolating a large quantity of the enzyme. Crystallized anthranilate synthase is useful to identify other agents that bind to and inhibit anthranilate synthase. The isolated enzyme could also be used to screen potential agents for efficacy of enzyme inhibition.

Once an anthranilate synthase gene of interest is isolated, an
25 efficient host vector system is necessary to introduce isolated genes or a group of genes into the genome of plant cells. The foreign genes should be expressed in the transformed plant cells and stably transmitted (somatically and sexually) to the next generation of cells produced. The vector should be capable of introducing, maintaining, and expressing a gene in plant cells, from a variety of
30 sources, including but not limited to plants and animals, bacteria, fungi, yeast or virus. Additionally, it should be possible to introduce the vector into a wide

variety of monocot and dicot plants. The new gene is passed on to progeny by normal breeding.

Introduction and expression of foreign genes in dicotyledonous (broad-leaved) plants such as tobacco, potato and alfalfa has been shown to be

- 5 possible using the T-DNA of the tumor-inducing (Ti) plasmid of *Agrobacterium tumefaciens*. Using recombinant DNA techniques and bacterial genetics, a wide variety of foreign DNAs can be inserted into T-DNA in *Agrobacterium*.
Following infection by the bacterium containing the recombinant Ti plasmid, the foreign DNA is inserted into the host plant chromosomes, thus producing a
- 10 genetically engineered cell and eventually a genetically engineered plant. A second approach is to introduce root-inducing (Ri) plasmids as the gene vectors.

While *Agrobacterium* appear to infect only dicots, many important crop plants including corn, wheat, rice, barley, oats, sorghum, millet, and rye are monocots and are not known to be susceptible to transformation by

- 15 *Agrobacterium*. The Ti plasmid, however, may be manipulated in the future to act as a vector for monocot plants. Additionally, using the Ti plasmid as a model system, it may be possible to artificially construct gene vectors for monocot plants. Ti-plasmids might also be introduced into monocots by artificial methods such as microinjection, or fusion between monocot protoplasts and
- 20 bacterial spheroplasts containing the T-region which can then be integrated into the plant nuclear DNA.

Transformation of plants with an anthranilate synthase may also be accomplished by introducing a DNA encoding an anthranilate synthase into other nucleic acid molecules that can transfer the inserted DNA into a plant

- 25 genome, e.g., plant pathogens such as DNA viruses like CaMV or geminiviruses, RNA viruses, and viroids; DNA molecules derived from unstable plant genome components like extrachromosomal DNA elements in organelles (e.g., chloroplasts or mitochondria), or nuclearly encoded controlling elements; DNA molecules from stable plant genome components (e.g., origins of replication and
- 30 other DNA sequences which allow introduced DNA to integrate into the organellar or nuclear genomes and to replicate normally, to autonomously

replicate, to segregate normally during cell division and sexual reproduction of the plant and to be inherited in succeeding generations of plants) or transposons.

DNA containing an anthranilate synthase gene may be delivered into plant cells or tissues directly by microorganisms with infectious plasmids, 5 infectious viruses, the use of liposomes, microinjection by mechanical or laser beam methods, by whole chromosomes or chromosome fragments, electroporation, and microparticle bombardment.

A. Strategy for Selection of Tryptophan Overproducer Cell Lines

10 Efficient selection of a desired tryptophan analog resistant, tryptophan overproducer variant using tissue culture techniques requires careful determination of selection conditions. These conditions are optimized to allow growth and accumulation of tryptophan analog resistant, tryptophan overproducer cells in the culture while inhibiting the growth of the bulk of the 15 cell population. The situation is complicated by the fact that the vitality of individual cells in a population can be highly dependent on the vitality of neighboring cells.

Conditions under which cell cultures are exposed to a tryptophan analog are determined by the characteristics of the interaction of the compound 20 with the tissue. Such factors as the degree of toxicity and the rate of inhibition should be considered. The accumulation of the compounds by cells in culture, and the persistence and stability of the compounds, both in the media and in the cells, also need to be considered. Additionally, it is important to determine whether the effects of the compounds can be readily reversed by the addition of 25 tryptophan.

The effects of the analog on culture viability and morphology is carefully evaluated. It is especially important to choose analog exposure conditions which have no impact on plant regeneration capability of cultures. Choice of analog exposure conditions is also influenced by whether the analog 30 kills cells or simply inhibits cell divisions.

The choice of a selection protocol is dependent upon the considerations described above. The protocols briefly described below may be utilized in the selection procedure. For example, to select for cells that are resistant to growth inhibition by tryptophan or an analog thereof, finely divided 5 cells in liquid suspension culture can be exposed to high tryptophan or analog levels for brief periods of time. Surviving cells are then allowed to recover and accumulate and are then reexposed for subsequently longer periods of time. Alternatively, organized partially differentiated cell cultures are grown and subcultured with continuous exposure to initially low levels of free L-tryptophan 10 or an analog thereof. Concentrations are then gradually increased over several subculture intervals. While these protocols can be utilized in a selection procedure, the present invention is not limited to these procedures.

B. Selection and Characterization of Resistant Cell Lines

15 Selections are carried out until cells or tissue are recovered which are observed to be growing well in the presence of normally inhibitory levels of tryptophan or an analog thereof. These cell "lines" are subcultured several additional times in the presence of tryptophan or the analog to remove non-resistant cells and then characterized. The amount of resistance which has been 20 obtained is determined by comparing the growth of these cell lines with the growth of unselected cells or tissue in the presence of various analog or free L-tryptophan concentrations. Stability of the resistance trait of the cultured cells may be evaluated by simply growing the selected cell lines in the absence of analog or free L-tryptophan for various periods of time and then analyzing 25 growth after re-exposing the tissue to the analog or free L-tryptophan.

The resistant cell lines may also be evaluated using *in vitro* chemical studies to verify that the site of action of the analog is altered to a form which is less sensitive to inhibition by tryptophan and/or an analog thereof.

C. Plant Regeneration and Production of Seed

Cell lines exhibiting satisfactory levels of resistance to the tryptophan analog or free L-tryptophan are put through a plant regeneration protocol to obtain mature plants and seed expressing the resistance trait. The 5 plant regeneration protocol allows the development of somatic embryos and the subsequent growth of roots and shoots.

Mature plants are then obtained from cell lines that are known to express the trait. If possible, the regenerated plants are self pollinated. In addition, pollen obtained from the regenerated plants is crossed to seed grown 10 plants of agronomically important inbred lines. In some cases, pollen from plants of these inbred lines is used to pollinate regenerated plants. The trait is genetically characterized by evaluating the segregation of the trait in first and later generation progeny. The heritability and expression in plants of traits selected in tissue culture are of particular importance if the traits are to be 15 commercially useful.

D. Development of Tryptophan Overproducer Commercial Hybrid Seed

The commercial value of tryptophan overproducer corn is greatest if many different hybrid combinations are available for sale. The farmer 20 typically grows more than one kind of hybrid based on such differences as maturity, standability or other agronomic traits. Additionally, hybrids adapted to one part of the corn belt are not adapted to another part because of differences in such traits as maturity, disease, and insect resistance. Because of this, it is necessary to breed tryptophan overproduction into a large number of parental 25 lines so that many hybrid combinations can be produced.

A conversion process (backcrossing) is carried out by crossing the original overproducer line to normal elite lines and crossing the progeny back to the normal parent. The progeny from this cross will segregate such that some plants carry the gene responsible for overproduction whereas some do not. 30 Plants carrying such genes will be crossed again to the normal parent resulting in progeny which segregate for overproduction and normal production once more.

This is repeated until the original normal parent has been converted to an overproducing line, yet possesses all other important attributes as originally found in the normal parent. A separate backcrossing program is implemented for every elite line that is to be converted to tryptophan overproducer line.

5 Subsequent to the backcrossing, the new overproducer lines and the appropriate combinations of lines which make good commercial hybrids are evaluated for overproduction as well as a battery of important agronomic traits. Overproducer lines and hybrids are produced which are true to type of the original normal lines and hybrids. This requires evaluation under a range of
10 environmental conditions where the lines or hybrids will generally be grown commercially. For production of high tryptophan corn, it may be necessary that both parents of the hybrid seed corn be homozygous for the high tryptophan character. Parental lines of hybrids that perform satisfactorily are increased and used for hybrid production using standard hybrid seed corn production practices.

15

E. Alternate Methods of Obtaining Variant Plants that Overproduce Tryptophan

Any method which can be utilized to improve the quality and quantity of free tryptophan in plants can be utilized. Generally, biosynthesis of
20 amino acids, including tryptophan, are controlled by feedback regulation. The two major types of feedback regulation are feedback (or end product) inhibition and feedback repression. In feedback inhibition, the final metabolite of the pathway inhibits the activity of an enzyme of the pathway. Feedback repression is the inhibition of formation of one or more enzymes in a pathway by an end
25 product or a derivative of the end product. For many amino acid biosynthetic pathways, the amino acid end product first combines with its transfer RNA (tRNA) to cause repression. Feedback regulation may be circumvented by (a) decrease in the concentration of an inhibitory or repressive end product or (b) mutational alteration of the enzyme or enzyme forming system to a condition
30 less sensitive to feedback effects, i.e., mutation to feedback resistance (For a

more complete review of this topic, see Demain, Advan. Biochem. Eng., 1, 113-141 (1971)).

Variants are isolated that may possess an enzyme resistant to feedback inhibition and/or feedback repression by using analogs of the amino acid that exert inhibition and/or repression but cannot be used for protein synthesis. Variants may be resistant to the analog due to an alteration in the structure of the feedback-inhibited enzyme (inhibition resistant) whereas others may have an altered enzyme-forming system (repression resistant).

Additionally, these variants overproduce the amino acid due to altered control mechanisms. Mutations to both types of resistance (double mutants) in one line may result in a marked increase in amino acid production.

Any alteration or replacement of anthranilate synthase which leads to deregulation of feedback regulation and overproduction of tryptophan in callus culture, tissue culture, seed and regenerated plants may be utilized in the present invention. Anthranilate synthase may be altered or replaced in any plant species; of especially great importance are the agronomic and horticulture crops which are normally deficient or produce low quantities of tryptophan. The alteration of anthranilate synthase may be accomplished by any of a variety of means, including but not limited to the following methods: (1) spontaneous variation and direct mutant selection in tissue cultures; (2) direct or indirect mutagenesis procedures on tissue cultures of all types, seeds and plants; and (3) isolation of genes, manipulation, modification, or synthesis in whole or part of genes using molecular biology, chemical technologies and state-of-the-art procedures and reintroduction of resistance genes into plants.

Thus, tryptophan overproducer plants may be developed using any of the methods described above including, but not limited to, conventional genetic and plant breeding procedures, whole plant genetic and transgenic methods, and somatic hybridization by protoplast fusion.

F. Formation of an Expression Cassette

An expression cassette of the invention can comprise a DNA molecule encoding an anthranilate synthase gene operably linked to a promoter functional in a host cell. The gene can code for an anthranilate synthase that is 5 substantially tolerant and/or resistant to inhibition by free L-tryptophan or an amino acid analog of tryptophan.

1. Isolation and Identification of a Gene Coding for an Anthranilate Synthase

10 A gene encoding an anthranilate synthase can be identified and isolated by standard methods, as described by Sambrook et al., Molecular Cloning: A Laboratory Manual, Cold Spring Harbor, NY (1989). The gene can also be obtained from tryptophan analog-tolerant cell lines such as plant cell lines, prepared as described in U.S. Patent No. 4,642,411, which is hereby 15 incorporated by reference.

A gene encoding an anthranilate synthase can be identified by screening of a DNA or cDNA library generated from nucleic acid derived from a particular cell type, cell line, primary cells, or tissue. Examples of libraries to identify and isolate a plant anthranilate synthase include, but are not limited to, a 20 cDNA library derived from inbred line B73 (Stratagene, La Jolla, California, Cat. #937005, Clontech, Palo Alto, California, Cat. # FL1032a, #FL1032b, and FL1032n), genomic library from inbred line Mo17 (Stratagene, Cat. #946102) or genomic library from inbred line B73 (Clontech, Cat. # FL1032d). Screening for DNA fragments that encode all or a portion of the gene encoding an anthranilate 25 synthase can be accomplished by screening plaques from a genomic or cDNA library for hybridization to a probe of an anthranilate synthase gene from other organisms or by screening plaques from a cDNA expression library for binding to antibodies that specifically recognize anthranilate synthase. DNA fragments that hybridize to anthranilate synthase probes from other organisms and/or 30 plaques carrying DNA fragments that are immunoreactive with antibodies to anthranilate synthase can be subcloned into a vector and sequenced and/or used as probes to identify other cDNA or genomic sequences encoding all or a portion

of the desired anthranilate synthase gene. Preferred cDNA probes for screening a maize or plant library can be obtained from plasmid clones pDPG600 or pDPG602.

In a preferred version, a maize anthranilate synthase gene is identified and isolated from a 5-methyltryptophan- or 6-methylanthranilate-tolerant plant cell line prepared as described in Examples 1, 3, and 4. A cDNA library can be prepared by oligo dT priming. Plaques containing DNA fragments can be screened with probes or antibodies specific for anthranilate synthase. DNA fragments encoding a portion of an anthranilate synthase gene can be subcloned and sequenced and used as probes to identify a genomic anthranilate synthase gene. DNA fragments encoding a portion of a maize anthranilate synthase can be verified by determining sequence homology with other known anthranilate synthase genes or by hybridization to anthranilate synthase-specific messenger RNA. Once cDNA fragments encoding portions of the 5', middle and 3' ends of a maize anthranilate synthase are obtained, they can be used as probes to identify and clone a complete genomic copy of a maize anthranilate synthase gene from a maize genomic library.

Portions of the genomic copy or copies of an anthranilate synthase gene can be sequenced and the 5' end of the gene identified by standard methods including either DNA sequence homology to other anthranilate synthase genes or by RNAase protection analysis, as described by Sambrook et al., Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Press, Cold Spring Harbor, New York (1989). Once portions of the 5' end of the gene are identified, complete copies of the anthranilate synthase gene can be obtained by standard methods, including cloning or polymerase chain reaction (PCR) synthesis using oligonucleotide primers complementary to the DNA sequence at the 5' end of the gene. The presence of an isolated full-length copy of the anthranilate synthase gene can be verified by hybridization, partial sequence analysis, or by expression of a maize anthranilate synthase enzyme. The anthranilate synthase gene cloned from a 5-MT- or 6-MA-resistant cell line can be assessed for tolerance to 5-MT, 6-MA, or other amino acid analogs of

tryptophan by standard methods, as described in U.S. Patent No. 4,581,847, issued April 15, 1986, the disclosure of which is incorporated by reference herein.

5 Anthranilate synthase genes resistant and/or tolerant to amino acid analogs of tryptophan can be obtained by several methods. The methods include, but are not limited to:

1. spontaneous variation and direct mutant selection in cultures;
2. direct or indirect mutagenesis procedures on tissue cultures of any cell types or tissue, seeds or plants; and
- 10 3. mutation of the cloned anthranilate synthase gene by methods such as site specific mutagenesis (Sambrook et al., cited *supra*), transposon mediated mutagenesis (Berg et al., Biotechnology, 1, 417 (1983)), and deletion mutagenesis (Mitra et al., Molec. Gen. Genetic., 215, 294 (1989)).

15 Mutants can be identified by a change in a functional activity of the enzyme encoded by the gene in the presence of free L-tryptophan or amino acid analogs of tryptophan, or by detecting a change in the DNA sequence using restriction enzyme mapping or DNA sequence analysis.

In a preferred version, a gene encoding a maize anthranilate synthase substantially tolerant to 5-methyltryptophan is isolated from a maize 5-methyltryptophan tolerant cell line. See U.S. Patent No. 4,581,847, issued April 15, 1986, the disclosure of which is incorporated by reference herein. Briefly, partially differentiated plant cell cultures are grown and subcultured with continuous exposures to low levels of 5-methyltryptophan. 5-methyltryptophan concentrations are then gradually increased over several subculture intervals. Cells or tissues growing in the presence of normally toxic 5-methyltryptophan levels are repeatedly subcultured in the presence of 5-methyltryptophan and characterized. Stability of the 5-methyltryptophan tolerance trait of the cultured cells may be evaluated by growing the selected cell lines in the absence of 5-methyltryptophan for various periods of time and then analyzing growth after exposing the tissue to 5-methyltryptophan.

Cell lines which are tolerant by virtue of having an altered anthranilate synthase enzyme can be selected by identifying cell lines having enzyme activity in the presence of normally toxic, i.e., growth inhibitor, levels of 5-methyltryptophan.

5 Cell lines with an anthranilate synthase of reduced sensitivity to 5-methyltryptophan inhibition can be used to isolate a 5-methyltryptophan-resistant anthranilate synthase. A DNA library from a cell line tolerant to 5-methyltryptophan can be generated and DNA fragments encoding all or a portion of an anthranilate synthase gene can be identified by hybridization to a cDNA

10 probe encoding a portion of an anthranilate synthase gene. A complete copy of the altered gene can be obtained either by cloning and ligation or by PCR synthesis using appropriate primers. The isolation of the altered gene coding for anthranilate synthase can be confirmed in transformed plant cells by determining whether the anthranilate synthase being expressed retains enzyme activity when

15 exposed to normally toxic levels of 5-methyltryptophan.

2. Promoters

Once an anthranilate synthase gene is obtained and amplified, it is operably combined with a promoter to form an expression cassette.

20 Most genes have regions of DNA sequence that are known as promoters and which regulate gene expression. Promoter regions are typically found in the flanking DNA sequence upstream from the coding sequence in both prokaryotic and eukaryotic cells. A promoter sequence provides for regulation of transcription of the downstream gene sequence and typically includes from

25 about 50 to about 2,000 nucleotide base pairs. Promoter sequences also contain regulatory sequences such as enhancer sequences that can influence the level of gene expression. Some isolated promoter sequences can provide for gene expression of heterologous genes, that is a gene different from the native or homologous gene. Promoter sequences are also known to be strong or weak or

30 inducible. A strong promoter provides for a high level of gene expression, whereas a weak promoter provides for a very low level of gene expression. An

inducible promoter is a promoter that provides for turning on and off of gene expression in response to an exogenously added agent or to an environmental or developmental stimulus. Promoters can also provide for tissue specific or developmental regulation. An isolated promoter sequence that is a strong 5 promoter for heterologous genes is advantageous because it provides for a sufficient level of gene expression to allow for easy detection and selection of transformed cells and provides for a high level of gene expression when desired.

The promoter in an expression cassette of the invention can provide for expression of anthranilate synthase from an anthranilate synthase 10 gene. Preferably, the gene is expressed so as to result in an increase in tolerance of the plant cells to feedback inhibition by free L-tryptophan or to growth inhibition by an amino acid analog of tryptophan or so as to result in an increase in the total tryptophan content of the cells. The promoter can also be inducible so that gene expression can be turned on or off by an exogenously added agent. 15 For example, a bacterial promoter such as the P_{lac} promoter can be induced to varying levels of gene expression depending on the level of isothiopropylgalactoside added to the transformed bacterial cells. It may also be preferable to combine the gene with a promoter that provides tissue specific expression or developmentally regulated gene expression in plants.

20 Specific promoters functional in plant cells include, but are not limited to, the 35S CaMV promoter, rice actin promoter, ubiquitin, and nopaline synthase (NOS) promoter. Currently, a preferred promoter for expression in monocots is the 35S CaMV promoter.

An anthranilate synthase gene can be combined with the promoter 25 by standard methods as described in Sambrook et al., cited *supra*. Briefly, a plasmid containing a promoter such as the 35S CaMV promoter can be constructed as described in Jefferson, Plant Molecular Biology Reporter, 5, 387 (1987) or obtained from Clontech Lab in Palo Alto, California (e.g., pBI121 or pBI221). Typically, these plasmids are constructed to provide for multiple 30 cloning sites having specificity for different restriction enzymes downstream from the promoter. A gene encoding an anthranilate synthase can be subcloned

downstream from the promoter using restriction enzymes to ensure that the gene is inserted in proper orientation with respect to the promoter so that the gene can be expressed. In a preferred version, a plant anthranilate synthase is operably linked to a 35S CaMV promoter in a plasmid. In a more preferred embodiment 5 of the invention, a maize anthranilate synthase gene is operably linked to a 35S CaMV promoter in a plasmid. Once an anthranilate synthase gene is operably linked to a promoter, the expression cassette so formed can be subcloned into a plasmid or other vectors.

10 3. Optional Sequences in the Expression Cassette

The expression cassette can also optionally contain other DNA sequences. The expression cassette can further be comprised of a chloroplast transit peptide encoding DNA sequence operably linked between a promoter and the DNA sequence encoding an anthranilate synthase (for a review of plastid 15 targeting peptides, see Heijne et al., *Eur. J. Biochem.*, **180**, 535 (1989); Keegstra et al., *Ann. Rev. Plant Physiol. Plant Mol. Biol.*, **40**, 471 (1989)). If the expression cassette is to be introduced into a plant cell, the expression cassette 20 can also contain plant transcriptional termination and polyadenylation signals and translational signals linked to the 3' terminus of a plant anthranilate synthase gene. The expression cassette can also optionally further comprise plasmid DNA.

An exogenous chloroplast transit peptide can be used which is not encoded within a native plant anthranilate synthase gene. A chloroplast transit peptide is typically 40 to 70 amino acids in length and functions post- 25 translationally to direct a protein to the chloroplast. The transit peptide is cleaved either during or just after import into the chloroplast to yield the mature protein. The complete copy of a gene encoding a plant anthranilate synthase may contain a chloroplast transit peptide sequence. In that case, it may not be necessary to combine an exogenously obtained chloroplast transit peptide 30 sequence into the expression cassette.

Exogenous chloroplast transit peptide encoding sequences can be obtained from a variety of plant nuclear genes, so long as the products of the genes are expressed as preproteins comprising an amino terminal transit peptide and transported into chloroplast. Examples of plant gene products known to

5 include such transit peptide sequences include, but are not limited to, the small subunit of ribulose biphosphate carboxylase, ferredoxin, chlorophyll a/b binding protein, chloroplast ribosomal proteins encoded by nuclear genes, certain heatshock proteins, amino acid biosynthetic enzymes such as acetolactate acid synthase, 3-enolpyruvylphosphoshikimate synthase, dihydrodipicolinate synthase, and the like. Alternatively, the DNA fragment coding for the transit peptide may be chemically synthesized either wholly or in part from the known sequences of transit peptides such as those listed above.

10

Regardless of the source of the DNA fragment coding for the transit peptide, it should include a translation initiation codon and an amino acid sequence that is recognized by and will function properly in chloroplasts of the host plant. Attention should also be given to the amino acid sequence at the junction between the transit peptide and the anthranilate synthase enzyme where it is cleaved to yield the mature enzyme. Certain conserved amino acid sequences have been identified and may serve as a guideline. Precise fusion of

15

20 the transit peptide coding sequence with the anthranilate synthase coding sequence may require manipulation of one or both DNA sequences to introduce, for example, a convenient restriction site. This may be accomplished by methods including site-directed mutagenesis, insertion of chemically synthesized oligonucleotide linkers, and the like.

25 Once obtained, the chloroplast transit peptide sequence can be appropriately linked to the promoter and an anthranilate synthase gene in an expression cassette using standard methods. Briefly, a plasmid containing a promoter functional in plant cells and having multiple cloning sites downstream can be constructed as described in Jefferson, cited *supra*. The chloroplast transit

30 peptide sequence can be inserted downstream from the promoter using restriction enzymes. An anthranilate synthase gene can then be inserted immediately

downstream from and in frame with the 3' terminus of the chloroplast transit peptide sequence so that the chloroplast transit peptide is linked to the amino terminus of the anthranilate synthase. Once formed, the expression cassette can be subcloned into other plasmids or vectors.

5 When the expression cassette is to be introduced into a plant cell, the expression cassette can also optionally include 3' nontranslated plant regulatory DNA sequences. The 3' nontranslated regulatory DNA sequence preferably includes from about 300 to 1,000 nucleotide base pairs and contains plant transcriptional and translational termination sequences. Examples of 3'

10 10 nontranslated regulatory DNA sequences functional in plant cells include, but are not limited to, about 500 base pairs of the 3' flanking DNA sequence of the pea ribulose biphosphate carboxylase small subunit E9 gene, the 3' flanking DNA sequence of the octopine synthase gene, and the 3' flanking DNA sequence of the nopaline synthase gene. These 3' nontranslated regulatory sequences can be

15 15 obtained as described in An, Methods in Enzymology, 153, 292 (1987) or are already present in plasmids available from commercial sources such as Clontech, Palo Alto, California. The 3' nontranslated regulatory sequences can be operably linked to the 3' terminus of an anthranilate synthase gene by standard methods.

20 20 An expression cassette of the invention can also be further comprise plasmid DNA. Plasmid vectors include additional DNA sequences that provide for easy selection, amplification, and transformation of the expression cassette in prokaryotic and eukaryotic cells, e.g., pUC-derived vectors such as pUC8, pUC9, pUC18, pUC19, pUC23, pUC119, and pUC120, pSK-derived

25 25 vectors, pGEM-derived vectors, pSP-derived vectors, or pBS-derived vectors. The additional DNA sequences include origins of replication to provide for autonomous replication of the vector, selectable marker genes, preferably encoding antibiotic or herbicide resistance, unique multiple cloning sites providing for multiple sites to insert DNA sequences or genes encoded in the

30 30 expression cassette, and sequences that enhance transformation of prokaryotic and eukaryotic cells.

Another vector that is useful for expression in both plant and prokaryotic cells is the binary Ti plasmid (as disclosed in Schilperoort et al., U.S. Patent No. 4,940,838, issued July 10, 1990) as exemplified by vector pGA582. This binary Ti plasmid vector has been previously characterized by An, cited *supra*, and is available from Dr. An. This binary Ti vector can be replicated in prokaryotic bacteria such as *E. coli* and *Agrobacterium*. The *Agrobacterium* plasmid vectors can be used to transfer the expression cassette to plant cells. The binary Ti vectors preferably include the nopaline T DNA right and left borders to provide for efficient plant cell transformation, a selectable marker gene, unique 5 multiple cloning sites in the T border regions, the *colE1* replication origin and a wide host range replicon. The binary Ti vectors carrying an expression 10 cassette of the invention can be used to transform both prokaryotic and eukaryotic cells, but is preferably used to transform plant cells.

15 G. **Method for Screening for Expression of an Anthranilate Synthase Gene**

A method for screening for expression of an anthranilate synthase gene is also provided by the invention. Once formed, an expression cassette 20 comprising an anthranilate synthase gene can be subcloned into a known expression vector. The screening method in the invention includes the steps of introducing an expression vector into a host cell and detecting and/or quantitating expression of an anthranilate synthase gene. This method of screening is useful to identify expression cassettes providing for an expression of 25 an anthranilate synthase gene, and expression of an anthranilate synthase in the chloroplast of a transformed plant cell.

Suitable known expression vectors include plasmids that autonomously replicate in prokaryotic and/or eukaryotic cells. Specific examples include plasmids such as pUC, pSK, pGEM, pBS and pSP-derived 30 vectors described above. the pBI121 or pBI221 plasmid constructed as described by Jefferson, cited *supra*, or a binary Ti plasmid vector such as pG582 as described by An, cited *supra*, and the like.

An expression cassette of the invention can be subcloned into an expression vector by standard methods. The expression vector can then be introduced into prokaryotic or eukaryotic cells by currently available methods including, but not limited to, protoplast transformation, *Agrobacterium*-mediated transformation, electroporation, microprojectile bombardment, tungsten whiskers (Coffee et al., U.S. Patent No. 5,302,523, issued April 12, 1994) and liposomes. The expression vector can be introduced into plant cells such as maize, tobacco, *Brassica*, Black Mexican sweet corn, and *Arabidopsis* cells. Plant cells useful for transformation include callus, embryos, meristematic tissue, gametic tissue, or cultured suspension cells.

The vector can also be introduced into prokaryotic cells such as *E. coli* or *Agrobacterium*. Transformed cells can be selected typically using a selectable marker encoded on the expression vector.

Marker genes are also useful in the present invention. Marker genes are genes that impart a distinct phenotype to cells expressing the marker gene and thus allow such transformed cells to be distinguished from cells that do not have the marker. Such genes may encode either a selectable or screenable marker, depending on whether the marker confers a trait which one can 'select' for by chemical means, i.e., through the use of a selective agent (e.g., a herbicide, antibiotic, or the like), or whether it is simply a trait that one can identify through observation or testing, i.e., by 'screening' (e.g., the R-locus trait). Of course, many examples of suitable marker genes are known to the art and can be employed in the practice of the invention.

Included within the terms selectable or screenable marker genes are also genes which encode a "secretable marker" whose secretion can be detected as a means of identifying or selecting for transformed cells. Examples include markers which encode a secretable antigen that can be identified by antibody interaction, or even secretable enzymes which can be detected by their catalytic activity. Secretable proteins fall into a number of classes, including small, diffusible proteins detectable, e.g., by ELISA; and proteins that are

inserted or trapped in the cell wall (e.g., proteins that include a leader sequence such as that found in the expression unit of extensin or tobacco PR-S).

With regard to selectable secretable markers, the use of a gene that encodes a protein that becomes sequestered in the cell wall, and which 5 protein includes a unique epitope is considered to be particularly advantageous. Such a secreted antigen marker would ideally employ an epitope sequence that would provide low background in plant tissue, a promoter-leader sequence that would impart efficient expression and targeting across the plasma membrane, and would produce protein that is bound in the cell wall and yet accessible to 10 antibodies. A normally secreted wall protein modified to include a unique epitope would satisfy all such requirements.

One example of a protein suitable for modification in this manner is extensin, or hydroxyproline rich glycoprotein (HPRG). The use of the maize HPRG (Steifel et al., The Plant Cell, 2, 785 (1990)) is preferred as this molecule 15 is well characterized in terms of molecular biology, expression, and protein structure. However, any one of a variety of extensins and/or glycine-rich wall proteins (Keller et al., EMBO J., 8, 1309 (1989)) could be modified by the addition of an antigenic site to create a screenable marker.

Elements of the present disclosure are exemplified in detail 20 through the use of particular marker genes, however in light of this disclosure, numerous other possible selectable and/or screenable marker genes will be apparent to those of skill in the art in addition to the one set forth hereinbelow. Therefore, it will be understood that the following discussion is exemplary rather than exhaustive. In light of the techniques disclosed herein and the general 25 recombinant techniques which are known in the art, the present invention renders possible the introduction of any gene, including marker genes, into a recipient cell to generate a transformed monocot.

Possible selectable markers for use in connection with the present invention include, but are not limited to, a *neo* gene (Potrykus et al., Mol. Gen. Genet., 199, 183 (1985)) which codes for kanamycin resistance and can be 30 selected for using kanamycin, G418, and the like; a *bar* gene which codes for

bialaphos resistance; a gene which encodes an altered EPSP synthase protein (Hinchee et al., *Biotech.*, **6**, 915 (1988)) thus conferring glyphosate resistance; a nitrilase gene such as *bxn* from *Klebsiella ozaenae* which confers resistance to bromoxynil (Stalker et al., *Science*, **242**, 419 (1988)); a mutant acetolactate synthase gene (ALS) which confers resistance to imidazolinone, sulfonylurea or other ALS-inhibiting chemicals (European Patent Application 154,204, 1985); a methotrexate-resistant DHFR gene (Thillet et al., *J. Biol. Chem.*, **263**, 12500 (1988)); a dalapon dehalogenase gene that confers resistance to the herbicide dalapon; or a mutated anthranilate synthase gene that confers resistance to 5-methyl tryptophan. Where a mutant EPSP synthase gene is employed, additional benefit may be realized through the incorporation of a suitable chloroplast transit peptide, CTP (European Patent Application 0,218,571, 1987).

An illustrative embodiment of a selectable marker gene capable of being used in systems to select transformants is the genes that encode the enzyme phosphinothricin acetyltransferase, such as the *bar* gene from *Streptomyces hygroscopicus* or the *pat* gene from *Streptomyces viridochromogenes* (U.S. patent application Serial No. 07/565,844, which is incorporated by reference herein). The enzyme phosphinothricin acetyl transferase (PAT) inactivates the active ingredient in the herbicide bialaphos, phosphinothricin (PPT). PPT inhibits glutamine synthetase, (Murakami et al., *Mol. Gen. Genet.*, **205**, 42 (1986); Twell et al., *Plant Physiol.*, **91**, 1270 (1989)) causing rapid accumulation of ammonia and cell death. The success of the inventors of the 07/565,844 application in using this selective system in conjunction with monocots was particularly surprising because of the major difficulties which have been reported in transformation of cereals (Potrykus, *Trends Biotech.*, **7**, 269 (1989)).

Screenable markers that may be employed include, but are not limited to, a β -glucuronidase or *uidA* gene (GUS) which encodes an enzyme for which various chromogenic substrates are known; an R-locus gene, which encodes a product that regulates the production of anthocyanin pigments (red color) in plant tissues (Dellaporta et al., in *Chromosome Structure and Function*,

pp. 263-282 (1988)); a β -lactamase gene (Sutcliffe, PNAS USA, 75, 3737 (1978)), which encodes an enzyme for which various chromogenic substrates are known (e.g., PADAC, a chromogenic cephalosporin); a *xy/E* gene (Zukowsky et al., PNAS USA, 80, 1101 (1983)) which encodes a catechol dioxygenase that

5 can convert chromogenic catechols; an α -amylase gene (Ikuta et al., Biotech., 8, 241 (1990)); a tyrosinase gene (Katz et al., J. Gen. Microbiol., 129, 2703 (1983)) which encodes an enzyme capable of oxidizing tyrosine to DOPA and dopaquinone which in turn condenses to form the easily detectable compound melanin; a β -galactosidase gene, which encodes an enzyme for which there are

10 chromogenic substrates; a luciferase (*lux*) gene (Ow et al., Science, 234, 856 (1986)), which allows for bioluminescence detection; or even an aequorin gene (Prasher et al., Biochem. Biophys. Res. Comm., 126, 1259 (1985)), which may be employed in calcium-sensitive bioluminescence detection, or a green fluorescent protein gene (Niedz et al., Plant Cell Reports, 14, 403 (1995)).

15 The presence of the *lux* gene in transformed cells may be detected using, for example, X-ray film, scintillation counting, fluorescent spectrophotometry, low-light video cameras, photon-counting cameras, or multiwell luminometry. It is also envisioned that this system may be developed for populational screening for bioluminescence, such as on tissue culture plates,

20 or even for whole plant screening.

Transient expression of an anthranilate synthase gene can be detected and quantitated in the transformed cells. Gene expression can be quantitated by RT-PCR analysis, a quantitative Western blot using antibodies specific for the cloned anthranilate synthase or by detecting enzyme activity in

25 the presence of tryptophan or an amino acid analog of tryptophan. The tissue and subcellular location of the cloned anthranilate synthase can be determined by immunochemical staining methods using antibodies specific for the cloned anthranilate synthase or subcellular fractionation and subsequent biochemical and/or immunological analyses. Sensitivity of the cloned anthranilate synthase

30 to agents can also be assessed. Expression cassettes providing for expression of an anthranilate synthase or anthranilate synthase tolerant to inhibition by an

amino acid analog of tryptophan or free L-tryptophan can then be used to transform monocot and/or dicot plant tissue cells and to regenerate transformed plants and seeds.

5 H. Method of Imparting Tolerance to an Amino Acid Analog of Tryptophan and/or Altering the Tryptophan Content in a Plant Cell or Tissue

The invention provides a method of conferring tolerance to

10 tryptophan or an amino acid analog of tryptophan to a plant cell or tissue. The method includes the steps of introducing an expression cassette comprising a DNA sequence coding for an anthranilate synthase wherein the anthranilate synthase is enzymatically active in the presence of concentrations of the analog or free L-tryptophan that inhibit the endogenous or native anthranilate synthase.

15 The DNA sequence is operably linked to a promoter whereby, in the cells of the plant tissue, the sequence is expressed in an amount effective to render the cells of the plant tissue substantially tolerant to tryptophan or an analog thereof.

For example, an effective amount of gene expression to render the cells of the plant tissue substantially tolerant to 5-MT or 6-MA is an amount that

20 provides for plant cell growth in about 33 to 300 μ M 5-MT or 6-MA, preferably about 75-250 μ M 5-MT or 6-MA. These are amounts of 5-MT or 6-MA which normally inhibit a native anthranilate synthase. The amounts of other tryptophan analogs effective to render cells of a plant substantially tolerant to the analog can be determined by methods well known in the art.

25 An expression cassette of the invention can be introduced by methods of transformation especially effective for monocots, including, but not limited to, microprojectile bombardment of immature embryos (U.S. patent application 08/249,458, filed May 26, 1994, incorporated by reference herein; U.S. Patent Application Serial No. 08/112,245, filed August 25, 1993,

30 incorporated by reference herein) or Type II embryogenic callus cells as described by W.J. Gordon-Kamm et al. (Plant Cell, 2, 603 (1990)), M.E. Fromm et al. (Bio/Technology, 8, 833 (1990)) and D.A. Walters et al. (Plant Molecular Biology, 18, 189 (1992)), or by electroporation of type I embryogenic calluses

described by D'Halluin et al. (The Plant Cell, 4, 1495 (1992)), or by Krzyzek (U.S. Patent No. 5,384,253, issued January 24, 1995).

Transformed cells can be selected for the presence of a selectable marker gene. Transient expression of an anthranilate synthase gene can be

5 detected in the transgenic embryogenic calli using antibodies specific for the cloned anthranilate synthase, or by RT-PCR analyses. Transformed embryogenic calli can be used to generate transgenic plants that exhibit stable inheritance of the transformed anthranilate synthase gene. Plant cell lines exhibiting satisfactory levels of tolerance to an amino acid analog of tryptophan

10 or free L-tryptophan are put through a plant regeneration protocol to obtain mature plants and seeds expressing the tolerance traits by methods well known in the art (for example, see U.S. Patent Application Serial No. 08/112,245; and Laursen et al., Plant Mol. Biol., 24, 51 (1994)). The plant regeneration protocol allows the development of somatic embryos and the subsequent growth of roots

15 and shoots. To determine that the tolerance trait is expressed in differentiated organs of the plant, and not solely in undifferentiated cell culture, regenerated plants can be assayed for the levels of tryptophan present in various portions of the plant relative to regenerated, non-transformed plants.

The transgenic plants produced herein are expected to be useful

20 for a variety of commercial and research purposes. Transgenic plants can be created for use in traditional agriculture to possess traits beneficial to the consumer of the grain harvested from the plant (e.g., improved nutritive content in human food or animal feed). In such uses, the plants are generally grown for the use of their grain in human or animal foods. However, other parts of the

25 plants, including stalks, husks, vegetative parts, and the like, may also have utility, including use as part of animal silage or for ornamental purposes.

Transgenic plants may also find use in the commercial

manufacture of proteins or other molecules, where the molecule of interest is extracted or purified from plant parts, seeds, and the like. Cells or tissue from

30 the plants may also be cultured, grown *in vitro*, or fermented to manufacture such molecules.

The transgenic plants may also be used in commercial breeding programs, or may be crossed or bred to plants of related crop species.

Improvements encoded by the recombinant DNA may be transferred, e.g., from corn cells to cells of other species, e.g., by protoplast fusion.

5 The regenerated plants are self-pollinated. Pollen obtained from the regenerated plants is crossed to seed-grown plants of agronomically important inbred lines or regenerated plants are self-pollinated. Conversely, pollen from plants of these inbred lines is used to pollinate regenerated plants. The genetics of the trait are then characterized by evaluating the segregation of
10 the trait in the first and later generation progeny.

Regenerated plants are repeatedly crossed to inbred corn plants in order to introgress the exogenously introduced or preselected anthranilate synthase gene into the genome of the inbred corn plants. This process is referred to as backcross conversion. When a sufficient number of crosses to the recurrent
15 inbred parent have been completed in order to produce a product of the backcross conversion process that is substantially isogenic with the recurrent inbred parent except for the presence of the introduced transgene, the plant is self-pollinated at least once in order to produce a homozygous backcross converted inbred containing the preselected transgene. Progeny of these plants
20 are true breeding and tryptophan analog resistance and concentrations of tryptophan in the backcross converted inbreds are compared to the recurrent parent inbred.

Alternatively, seed from transformed monocot plants regenerated from transformed tissue cultures is grown in the field and self-pollinated to
25 generate true breeding plants. Progenies from these plants become true breeding lines which are evaluated for tryptophan amino acid analog tolerance in the field under a range of environmental conditions. Tryptophan amino acid analog tolerance must be sufficient to protect the monocot plants at the maximum delivery rate under field conditions which causes the amino acid analog to be
30 most active. The determination of appropriate concentrations of these agents and methods of application are well known in the art.

In a preferred version, an expression cassette comprised of a maize anthranilate synthase gene isolated from a maize cell line tolerant to 5-MT and linked to the 35S CaMV promoter is introduced into an 5-MT sensitive monocot tissue using biolistic transformation. Transformed calli are selected 5 and used to generate transgenic plants. Transformed calli and transgenic plants can be evaluated for tolerance to 5-MT or 6-MA and for stable inheritance of the tolerance trait.

The invention also provides a method of altering the tryptophan content in a plant cell or tissue. The method include the steps of introducing an 10 expression cassette comprising a DNA sequence coding for an anthranilate synthase that is resistant to inhibition by free L-tryptophan or an amino acid analog of tryptophan, operably linked to a promoter functional in a plant cell into the cells of plant, and expressing the gene in an amount effective to alter the tryptophan content of the plant cell. An alteration in the tryptophan content of a 15 plant cell can include a change in the total tryptophan content over that normally present in that type of plant cell not transformed with the expression cassette. Expression of an anthranilate synthase gene in an effective amount is that amount that may provide a change in the tryptophan content of the cell from about 1.1- to 50-fold over that normally present in the plant cell, and preferably 20 increases the amount of tryptophan of about 2- to 20-fold over the amount of tryptophan normally present in that plant cell.

To provide for expression of the anthranilate synthase gene, the gene can be combined with a promoter that provides for a high level of gene expression in plant cells, such as the 35S CaMV promoter.

25 An expression cassette as described above can be introduced into either monocots or dicots. An expression cassette can be introduced by standard methods including protoplast transformation, *Agrobacterium*-mediated transformation, microprojectile bombardment, electroporation, and the like. Transformed cells or tissues can be selected for the presence of a selectable 30 marker gene.

Transient expression of an anthranilate synthase gene can be detected in transformed cells or tissues by immunoreactivity with antibodies specific for anthranilate synthase. Stable expression of an anthranilate synthase can be detected by quantitative Western blots. A change in specific activity of 5 the enzyme in the presence of inhibitory amounts of tryptophan or an analog thereof can be detected by measuring enzyme activity in the transformed cells as described by Widholm, *Biochimica et Biophysica Acta*, 279, 48 (1972). A change in total tryptophan content can also be examined by standard methods as described by Jones et al., *Analyst*, 106, 968 (1981).

10 Transgenic plants and seeds can be generated from transformed cells and tissues showing a change in tryptophan content or in resistance to a tryptophan analog using standard methods. It is especially preferred that the tryptophan content of the leaves, seeds, or fruits is increased.

15 I. **Method of Introducing and Producing Anthranilate Synthase**

The invention also provides a method of introducing and producing anthranilate synthase in a host cell. The method includes the steps of introducing an expression cassette comprised of a gene encoding an anthranilate synthase into a host cell and identifying a cell with the expression cassette and/or 20 a cell expressing the gene. The availability of large amounts of purified enzyme provides for screening of the efficacy of such agents.

An expression cassette can include a promoter that is functional in either a eukaryotic or prokaryotic cell. The expression cassette can be introduced into a prokaryotic cell such as *E. coli*, or a eukaryotic cell such as a 25 plant or yeast. The preferred cell is a prokaryotic cell used routinely in producing recombinant proteins such as *E. coli*. Prokaryotic or eukaryotic transformed cells can be selected by standard methods.

Anthranilate synthase can be isolated from bacterial cells using standard methods, e.g., see Sambrook et al., *Molecular Cloning: A Laboratory 30 Manual*, (1989). The purified anthranilate synthase can then be characterized by standard methods.

J. Commercial Approaches to Tryptophan Extraction from High Tryptophan Maize Seed

Fertile, transgenic plants may then be used in a conventional maize breeding program in order to incorporate the introduced DNA into the 5 desired lines or varieties. Methods and references for convergent improvement of corn are given by Hallauer et al., In: Corn and Corn Improvement, Sprague et al. (eds.), pp. 463-564 (1988), incorporated herein by reference. Among the approaches that conventional breeding programs employ is a conversion process (backcrossing). Briefly, conversion is performed by crossing the initial 10 transgenic fertile plant to elite inbred lines. The progeny from this cross will segregate such that some of the plants will carry the recombinant DNA whereas some will not. The plants that do not carry the DNA are then crossed again to the elite inbred lines resulting in progeny which segregate once more. This backcrossing process is repeated until the original elite inbred has been 15 converted to a line containing the recombinant DNA, yet possession all important attributes originally found in the parent. Generally, this will require about 6-8 generations. A separate backcrossing program will be generally used for every elite line that is to be converted to a genetically engineered elite line.

Generally, the commercial value of the transformed corn 20 produced herein will be greatest if the recombinant DNA can be incorporated into many different hybrid combinations. A farmer typically grows several hybrids based on differences in maturity, standability, and other agronomic traits. Also, the farmer must select a hybrid based upon his or her geographic location since hybrids adapted to one region are generally not adapted to another 25 because of differences in such traits as maturity, disease, and insect resistance. As such, it is necessary to incorporate the introduced DNA into a large number of parental lines so that many hybrid combinations can be produced containing the desirable DNA.

Corn breeding and the techniques and skills required to transfer 30 genes from one line or variety to another are well known to those skilled in the art. Thus, introducing recombinant DNA into any other line or variety can be accomplished by these breeding procedures.

There are a number of methods that can be used to extract the free tryptophan from high tryptophan maize, however, the more economical of these would likely involve extracting the tryptophan prior to or following standard wet or dry milling processes (Watson, Corn and Corn Improvements, G.F. Sprague, 5 ed., Amer. Soc. of Agronomy, Madison, Wisconsin (1977)). This permits the value of the tryptophan to be added to the value of milled products.

In the wet milling process, one of the initial steps involves treating the kernels with an acidic aqueous solution and allowing a limited amount of lactic acid fermentation to occur. This treatment conditions and 10 softens the kernel and solubilizes many small molecules in the kernel including free amino acids. This acid environment will, however, result in breakdown of most of the solubilized tryptophan. Thus, to obtain significant amounts of tryptophan, it is preferred to extract the tryptophan prior to the acid treatment step. A method that can be used to carry out this pre-extraction process is 15 described below.

Maize seed can be processed in a series of vats at elevated temperatures (approximately 150°F) in an aqueous environment at basic/neutral pH (pH < 10). Water is added to the corn that has been extracted the longest in the process and flows in a countercurrent manner towards the newly introduced 20 seed. Following several days of extraction, the corn is then transferred to a standard wet milling plant. The liquid from the extraction process can then be filtered to remove solids then treated by standard chemical means, solvent extraction/phase separation, ion exchange chromatography and crystallization, to concentrate and purify the tryptophan. The degree of purification will depend on 25 the type of product desired--feed supplement, chemical feed stock, reagent chemical, etc.

In the dry milling processes, corn kernels are cleaned, brought to 20-22% moisture then milled, pressed, and sorted to give a variety of fractions--hominy feed, flaking, medium and fine grits, meal, and flour. One or more of 30 these fractions can then be extracted with water under neutral to basic conditions at elevated temperature to obtain the tryptophan. Again, the tryptophan can then

be concentrated and purified by standard chemical methods including solvent extraction/phase separation, ion exchange chromatography and crystallization.

Tryptophan may also be recovered by other conventional procedures. For example, a method for recovery is represented in U.S. Patent

5 No. 3,759,790 which is hereby incorporated by reference into the present specification.

EXAMPLE 1

Identification of Recombinant DNA Clones Corresponding to Genes Encoding Maize Anthranilate Synthase Alpha- and Beta-Subunits.

10 To obtain clones corresponding to maize genes encoding the alpha-subunit of anthranilate synthase, gene-specific oligonucleotide primers corresponding to the *Arabidopsis* ASA2 gene sequence (GenBank Accession M92354) were designed to allow for PCR amplification of a region from exons 10 through 11 of this gene in *Arabidopsis*. Exons 10 and 11 of the alpha subunit

15 of the *Arabidopsis* anthranilate synthase have been defined by Niyogi and Fink (*supra*). These primers, designated ASA2-C (AAGAAGATCTAATGCTGGAAAAA, SEQ ID NO:5) and ASA2-D (GATATTGTTGATGTGAGGTGTGA, SEQ ID NO:6), were used in a PCR amplification of *Arabidopsis* DNA under the following conditions: 0.5 µg of

20 DNA was used as template in reactions containing 0.5 µM each of primer ASA2-C and ASA2-D, 200 µM each dATP, dCTP, dGTP, and dTTP, 1 mM MgCl₂, 50 mM KCl, 10 mM Tris-HCl, pH 8.3, and 2.5U Taq DNA polymerase. Thermal cycling conditions were as follows: initial denaturation at 97°C for 2 minutes; three cycles of 97°C for 1 minute, 53°C for 1 minute, and 72°C for 1 minute; 33

25 cycles of 94°C for 1 minute, 53°C for 1 minute, and 72°C for 1 minute; and a final extension of 72°C for 4 minutes. A PCR-generated amplification product of 1037 base pairs was cleaved with NcoI to yield two fragments of 685 and 352 base pairs, the latter of which corresponds exclusively to exon 11 of *Arabidopsis* ASA2, from positions 5362 to 5714 of the M92354 sequence. This fragment

30 was gel-purified and used as a radiolabelled probe for screening a cDNA library constructed in the cloning vector LambdaZAP (Stratagene, La Jolla, California)

corresponding to mRNAs obtained from maize embryos (inbred line Va26) at 27 days after pollination (Belanger and Kriz, *Plant Physiol.*, **91**, 636 (1989)).

Initial screening of the library was performed under low-stringency hybridization conditions (42°C, 6XSSC, 1X Denhardt's solution, 5 0.1% SDS, 10 µg/ml sonicated calf thymus DNA). Filters were washed in 3XSSC, 0.1% SDS at room temperature, then in 1XSSC, 0.1% SDS, and finally in 1XSSC at 60°C. Subsequent rounds of screening for plaque purification purposes were performed in a similar manner except that a temperature of 48°C was used for the hybridization step. A total of five putative anthranilate synthase 10 cDNA clones were identified by this screening protocol.

Each of these clones was subjected to a limited amount of nucleotide sequence analysis using vector-specific primers and standard protocols (see, Sambrook et al., *supra*). Comparison of these nucleotide sequences indicated that four of the clones (pcASA2 1-1, 2-1, 4-1, and 5-3) were 15 identical in sequence and another (pcASA2 3-1) was similar, but not identical, to the others. These results indicated that cDNA clones corresponding to two distinct genes had been obtained by screening the library with a probe derived from the *Arabidopsis* anthranilate synthase gene sequence. Comparison of the gene sequences of these clones with the sequences of the *Arabidopsis* 20 anthranilate synthase genes (GenBankM92354 and M92355) revealed similarity between the maize and *Arabidopsis* sequences.

To obtain genomic clones corresponding to maize anthranilate synthase alpha subunits, a library of DNA fragments generated by partial digestion of nuclear DNA from the maize inbred line B73 was constructed in the 25 lambda cloning vector EMBL3 using standard protocols. This library was screened with a radiolabelled probe consisting of a 351 base pair Van91I fragment obtained from the clone pcASA2 2-1 (Figure 1). Hybridization was performed overnight at 68°C in a solution of 6XSSC, 0.1% SDS, 1X Denhardt's solution, and 10 µg/ml sonicated salmon sperm DNA. Washing of filters was 30 performed at 68°C in 2XSSC, 0.1% SDS. A single lambda clone was selected for analysis. DNA from this clone was subjected to digestion with a variety of

restriction enzymes followed by Southern blot analysis in which a 440 base pair Sall/EcoRI fragment from the 5' region of pcASA2 2-1 (Figure 1) was used as probe. This analysis identified a 5.9 kilobase pair EcoRI fragment, which was subsequently cloned into the plasmid vector pBSK (Stratagene, La Jolla,

5 California). This clone was designated pDPG668.

To obtain cDNA clones corresponding to the beta-subunit of anthranilate synthase, an oligonucleotide 40 bases in length (ASB-1, TGCATT GGAGAGGCATTGGATGAAAGATTATCCGTGCTC, SEQ ID NO:7) was designed to match the MZEORFH (GenBank M95067) sequence. The

10 MZEORFH sequence corresponds to a randomly-isolated maize cDNA which was shown to exhibit sequence similarity to the *Arabidopsis* ASB1 gene (GenBank L22585; Niyogi et al., *Plant Cell*, 5, 1011 (1993)). The ASB-1 oligonucleotide was end-labelled with 32 P-ATP using polynucleotide kinase as recommended by the manufacturer (BRL). Screening of the maize embryo
15 cDNA library in the LambdaZAP vector with radiolabelled ASB-1 as probe was performed as described for isolation of genomic clones corresponding to the alpha-subunit of anthranilate synthase. A single clone, designated pcASB1-4, was obtained by this screening protocol. A limited amount of sequence information was obtained from the ends of this clone using vector-specific
20 primers, and an internal portion of the sequence was obtained using the ASB-1 oligonucleotide as a sequencing primer. The internal nucleotide sequence obtained clone ASB1-4 exhibited 71% sequence identity to the *Arabidopsis* ASB1 gene sequence over a region of 93 nucleotides (positions 670 to 763 of GenBank L22585) (SEQ ID NO:8, Figure 6).

EXAMPLE 2

Genetic Mapping of the 5-MT Resistance Trait in Maize and cDNA Sequences Corresponding to Genes Encoding Anthranilate Synthase Sequences

5 The 5-MT resistance trait present in the maize line C28 was localized to the short arm of chromosome 1 using waxy reciprocal translocations. C28 plants homozygous for the 5-MT resistance trait were crossed by a series of waxy translocation stocks obtained from the Maize Genetics Stock Center, Urbana, Illinois. F1 plants obtained from these crosses were subsequently 10 crossed to a homozygous waxy tester stock, and progeny from this testcross were separated into starchy [Wx/(?)] and waxy [wx/wx] kernels using routine iodine staining procedures. Kernels of each class were subsequently subjected to a root growth bioassay for 5-methyltryptophan resistance as described by Hibberd et al., U.S. Patent No. 4,581,847. Linkage between the 5-MT resistance trait and 15 Wx was observed in progeny obtained from initial crosses involving wx stock T1-9c: of 91 Wx kernels analyzed, 77 were resistant to 5-MT and 14 were sensitive to 5-MT; of 104 wx/wx kernels analyzed, 28 were resistant to 5-MT and 76 were sensitive to 5-MT.

Linkage to markers on the short arm of chromosome 1 was 20 established by crossing a tester stock (sr1/sr1 zb4/zb4 P1-WW) with a C28 line homozygous for 5-MT resistance, and subsequent testcrossing of the F1 progeny back to the tester stock. The resultant progeny were scored for 5-MT resistance and for the presence of striate leaves. Of 134 individuals examined, 61 were classified as 5-MT resistant, normal leaves; 7 as 5-MT resistant, striate leaves; 9 25 as 5-MT sensitive, normal leaves, and 57 as 5-MT sensitive, striate leaves. These data indicated that the 5-MT locus defined by the C28 mutation is approximately 12 centimorgans (cm) from the sr1 locus on the short arm of chromosome 1.

The map positions of genes corresponding to the maize 30 anthranilate synthase cDNA clones were determined to ascertain whether any of these loci map near the 5-Mtr locus defined by the C28 mutation. Three

different probes which were specific for the three ASA2 cDNA clones (ASA2 1-1, ASA2 2-1, and ASA2 3-1) and the pcASB1-4 clone were mapped using a Recombinant Inbred mapping population of maize plants developed to facilitate placement of genes on the maize genetic map (Burr et al., *Genetics*, 118, 519 (1988)). The results are as follows:

Table 1

| Probe | Location ^a |
|-------|-----------------------|
| ASA2 | 3-11L253 |
| ASA2 | 3-19SASA2 2-11S073.5 |
| ASA2 | 1-11S073.5 |
| ASB1 | 42S087 |
| ASB1 | 410L080 |
| ASB1 | 49L083.6 |
| ASB1 | 42L156 |
| Ts21 | S085 |

^aLocation refers to chromosome number, arm, and map units from the first probe on the short arm as mapped on the 1994 RI map (Maize Genetics Newsletter, 68, 198 (1994)).

These data indicate that pcASA2 2-1 and pcASA2 1-1 map approximately 12 cm distal to the Ts2 locus, a locus mapping to the short arm of chromosome 1. Since sr1 and Ts2 are separated by 24 units on the morphological map, the location of anthranilate synthase sequences in this region of the genome is consistent with the location of the 5-Mtr trait. In addition, since nucleotide sequence data of clones pcASA2 2-1 and pcASA2 1-1 indicated that these two clones are identical in sequence, and mapping data indicated they are derived from the same region of the maize genome, it was concluded that these two clones correspond to the same anthranilate synthase gene and that this gene corresponds to the 5-MT locus defined by the C28 mutation. The clone pcASA2

3-1 mapped to loci on two other chromosome arms, and the pcASB1-4 clone mapped to loci on four other chromosome arms. These latter two clones therefore do not correspond to loci that are modified in the C28 line.

EXAMPLE 3

5 **Characterization of DNA Sequences Corresponding to Maize Genes Encoding Anthranilate Synthase Alpha Subunits**

Of the four clones (pcASA2 1-1, 2-1, 4-1, 5-3) which exhibited sequence identity for the regions initially analyzed, pcASA2 2-1 was determined to be the longest clone by restriction enzyme mapping analysis of the clones.

10 Thus, pcASA2 2-1 was selected for further analysis. The entire nucleotide sequence of the maize cDNA contained in the clone pcASA2 2-1, designated below as pDPG600 was determined using standard procedures employing the Sequenase system (USB, Cleveland, Ohio). Sequence analysis was performed using oligonucleotide primers specific for the cloning vector, pBSK, to obtain 15 sequence information from the ends of the cDNA fragment, and oligonucleotide primers specific for internal sequences based on internal sequence information from subclones generated from pcASA2 2-1 which were cloned in pBSK with endpoints at the EcoRV site shown in Figure 1. The sequence information obtained through the use of vector-specific primers was extended by the use of 20 oligonucleotide primers based on the pDPG600 nucleotide sequence and "walking" along the length of the clone in both directions. Sequence analysis and oligonucleotide primer design was performed using the GeneWorks (Intellegentics, Inc, Mountain View, California) and Oligo (National Biosciences, Plymouth, Minnesota) software programs, respectively.

25 The 5' end of the ASA2 transcript was identified by primer extension reactions in which reverse transcriptase was used to extend the oligonucleotide primer PE-1 (SEQ ID NO:9) using RNA isolated from developing maize embryos as a template, in the presence of a radiolabelled deoxynucleotide triphosphate under standard reaction conditions (Sambrook et 30 al., Molecular Cloning: A Laboratory Manual, (1989)). Comparison of the size of the primer extension product with a sequencing ladder generated from the

pDPG668 genomic clone with the PE-1 primer indicated that the 5' end of the transcript corresponds to a position 65 base pairs upstream of the 5' end of the pDPG600 cDNA clone. There is no ATG between the 5' end of the transcript and the first ATG in the cDNA sequence, indicating that the maize anthranilate synthase transcript corresponding to pDPG600 has a 5' untranslated region (UTR) of 142 nucleotides.

The cDNA insert in pDPG600 is 2060 base pairs in length and contains an open reading frame (ORF) of 1845 nucleotides (Figure 2, SEQ ID NO:1). This ORF, the 5' end of which represents the initial ATG in the

10 pDPG600 sequence, corresponds to a polypeptide of 615 amino acids with a predicted molecular mass of 67.8 kilodaltons. Previous work has indicated that most enzymes involved in biosynthesis of aromatic acids are localized in plastids (Zhao et al., *J. Biol. Chem.*, **270**, 6081 (1995)). Because the genes encoding these biosynthetic enzymes are nuclear-encoded genes, the targeting of the gene product to the plastid is through the presence of a transit peptide at the 5' end of the coding sequence. The predicted polypeptide encoded by pDPG600 (SEQ ID NO:2) contains a putative transit peptide cleavage sequence (VKC▼S) at amino acid residues 44-47, which is consistent with the consensus transit peptide cleavage signal of (V/I)X(A/C)▼A as described by Gavel and Von Heijne, *FEBS Lett.*, **261**, 455 (1990)). The actual amino acid sequence at the N-terminus of the maize anthranilate synthase enzyme has not yet been determined. If amino acid residues 1-46 function as a transit peptide, and if this sequence is removed during transport into the plastid, a polypeptide of 569 amino acids with molecular mass of 63.2 kilodaltons would be expected.

25 To determine the size of the mature anthranilate synthase alpha-subunit encoded by clone pDPG600, polyclonal antibodies were generated to a portion of the polypeptide encoded by the pDPG600 cDNA sequence. An EcoRV/XbaI restriction fragment from pDPG600, corresponding to nucleotide positions 1093 to 2017 of the pDPG600 sequence, was inserted into the protein 30 expression vector pProEx-1 (BRL) which had been cleaved with StuI and XbaI, restriction enzymes which have recognition sites in the multiple cloning site of

the vector. Both EcoRV and StuI generate blunt ends. This resulted in a clone that contained codons 344 through 615 of pDPG600 3' to the pProEx-1 leader sequence containing the 6xHis tag sequence. This clone contains 3' to the pDPG600 sequence a termination codon and a 103 base pair UTR. This clone

5 was transformed into DH α 5. Expression of the recombinant fusion protein was induced by 0.6 mM isopropyl- β -D-thiogalactoside. The presence of the 6xHis tag in the recombinant fusion protein allowed for affinity purification of the recombinant fusion protein using a nickel nitrilo-tri-acetic acid resin as recommended by the manufacturer, BRL (see Döbeli et al., U.S. Patent

10 No. 5,284,933, issued February 8, 1994, incorporated by reference herein).

A total of 7.8 mg of the 33.5 kD recombinant fusion protein, estimated to be approximately 95% pure by SDS-PAGE analysis was obtained. A total of 4 mg of this protein was provided to HTI Bio Products, Inc. (Ramona, California) for production of polyclonal antibodies in rabbits. Analysis of maize

15 protein extracts by SDS-PAGE and immunoblotting with this polyclonal rabbit sera revealed the presence of a 63 kD polypeptide in these extracts. This data strongly suggests that the mature maize anthranilate synthase enzyme has a molecular mass consistent with the cleavage of a ca. 4 kilodalton transit peptide sequence.

20 The amino acid sequence predicted from the pDPG600 nucleotide sequence exhibits a significant amount of homology to sequences of other isolated genes encoding anthranilate synthase, particularly to those of *Arabidopsis* (Figure 3). The maize anthranilate synthase predicted amino acid sequence exhibits 63% identity to each of the *Arabidopsis* anthranilate synthase

25 alpha subunits encoded by the ASA1 (SEQ. ID NO:3) and ASA2 (SEQ. ID NO:4) genes. As shown in Figure 3, similarity between the maize and the *Arabidopsis* ASA2 sequences is apparent after amino acid position 101 in the pDPG600 predicted amino acid sequence. These similarities clearly indicate that pDPG600 corresponds to a maize homologue of the *Arabidopsis* anthranilate

30 synthase gene ASA2.

EXAMPLE 4

Identification of a Single Base-Pair Difference in Anthranilate Synthase cDNA Sequences Obtained from Maize Plants Homozygous for the 5-MT Resistance Trait

5 As described above, the maize anthranilate synthase gene represented by pDPG600 maps to the same region of the genome as does the 5-MT resistance trait, strongly suggesting that modification of this gene in the C28 cell line led to decreased sensitivity with respect to tryptophan feedback inhibition. Analysis of *S. typhurium* anthranilate synthase mutants in the subunit 10 encoded by the *TrpE* gene (Caligiuri and Bauerle, *J. Biol. Chem.*, **266**, 8328 (1991)) identified two regions of the anthranilate synthase polypeptide involved in feedback inhibition. Although most of these mutant polypeptides exhibited a decreased affinity for tryptophan with no change in substrate or catalytic activity, an 18 amino acid region of *TrpE* was identified that is important for tryptophan 15 regulation. This region was chosen for analysis of anthranilate synthase sequences from 5-MT resistant plants.

Nucleotide sequence information from pDPG600 was used to design a set of primers, A21-8 (SEQ ID NO:10) and A21-9 (SEQ ID NO:11), which would amplify a significant portion of the gene, including the 18 amino 20 acid region identified by *TrpE* mutant analysis. These primers were used in reverse transcriptase-PCR (RT-PCR) assays of RNA obtained from 5-MT resistant plants (leaves, developing embryos, developing endosperm, and germinating embryos). The RT-PCR assays were performed using the reagents supplied by Perkin-Elmer Cetus in the GeneAmp RNA PCR Kit as follows: 1 µg 25 of RNA was subjected to a 20 µl reverse transcription reaction containing 1 mM each dATP, dCTP, dGTP, and dTTP, 5 mM MgCl₂, 50 mM KCl, 10 mM Tris-HCl, pH 8.3, 50U MMLV reverse transcriptase, and 2.5 µM oligo d(T)₁₆. Reactions were performed at 42°C for 15 minutes followed by a 5 minute 30 incubation at 99°C. Samples were then subjected to PCR in 100 µl reactions containing 0.25 µM of each primer A21-8 and A21-9, 200 µM each dATP, dCTP, dGTP, and dTTP, 1 mM MgCl₂, 50 mM KCl, 10 mM Tris-HCl, pH 8.3,

and 2.5U Taq DNA polymerase. Reactions were performed in the wells of microtiter plates in an MJ Research thermal cycler (Model PTC-100) with a 96 well plate block. Thermal cycling conditions were as follows: initial denaturation at 97°C for 2 minutes; 3 cycles of 97°C for 1 minute, 56°C for 1 minute, and 72°C for 1 minute; 33 cycles of 94°C for 1 minute, 56°C for 1 minute, and 72°C for 1 minute; and a final extension of 72°C for 4 minutes. All samples assayed yielded the expected size band of 700 base pairs as predicted from the pDPG600 sequence.

To insert these sequences into a plasmid vector, the "TA" cloning technique, which takes advantage of the fact that the Taq polymerase used during PCR amplification adds a single dA residue onto the 5' ends of amplification products (Holton and Graham, NAR, 19, 1156 (1991)), was used. The plasmid vector pBSK was cleaved with EcoRV to generate blunt ends and subsequently modified by the addition of a single dT residue to each resultant 3' end through the action of terminal deoxynucleotide transferase. Ligation of the RT-PCR products to the T-tailed vector was achieved through association of the 5' A overhang in the amplification products with the 3' T overhang on the vector and the activity of T4 DNA ligase. Ligation reactions were used to transform competent bacteria. DNA from individual bacterial clones was then subjected to sequence analysis to examine the region corresponding to the residues implicated in 5-MT resistance. Both strands of four clones, each from a different RNA source, were sequenced. Each of these clones (from leaf, 24 DAP endosperm, 24 DAP embryo, or 2 day germinating embryo from homozygous C28 plants) were identical in sequence, and each of these four clones contained a single base substitution (T to A) corresponding to position 1194 in the pDPG600 sequence. This nucleotide substitution results in a methionine to lysine residue substitution at codon position 377 relative to the initiator methionine codon. This residue is one which is implicated in feedback inhibition through *S. typhimurium* *TrpE* mutant analysis. Other residues in the *TrpE* gene have been implicated as being involved in tryptophan feedback regulation, as indicated in the maize and *Arabidopsis* sequences shown in Figure 3. It is likely that amino acid

substitutions at these other residues will also result in a modification of tryptophan feedback sensitivity of an anthranilate synthase.

To generate cDNA clones containing the entire protein coding region of maize anthranilate synthase with the met to lys substitution at codon

5 position 377, a region from one of the RT-PCR-amplified clones (pcASA2m4) with the 5-MT resistance allele was substituted for the corresponding region of the wild-type pcASA2 1-1 clone. This was accomplished by cleaving both pDPG600 and pcASA2m4 with the restriction enzymes NsiI and Van91I to generate a 403 base pair NsiI/Van91I fragment with the C28 mutation (SEQ. ID 10 NO:12) and a 351 base pair Van91I fragment, and, in the case of pDPG600, a 4258 base pair NsiI/Van91I fragment which contains the 5' region upstream of the NsiI site and the 3' region downstream from the second Van91I site fused to the pBSK vector. The 403 base pair NsiI/Van91I fragment from pcASA2m4 was isolated and used in a three-way ligation reaction which also contained the 15 351 base pair Van91I fragment and the 4258 base pair NsiI/Van91I fragment from pDPG600. Because the five residues within the two Van91I recognition sites of pDPG600 are different from each other, the only recombinant DNA molecules which could be obtained from such a three-way ligation are those that reconstruct the original sequences within the Van91I sites. A total of 10 clones 20 obtained from this three-way ligation were selected and screened for the presence of the NsiI site and the two Van91I sites. All 10 clones contained each of these sites. Two clones were selected for nucleotide sequence analysis with the oligonucleotide primer A21-8 (SEQ ID NO:10), and both clones were found to contain the T to A substitution at position 1194. This confirmed that a 25 recombinant DNA clone corresponding to a full-length anthranilate synthase cDNA clone, but which differed from the wild type sequence by a single nucleotide and which changed the codon at position 377 from a methionine codon to a lysine codon, had been obtained. This clone was designated pDPG602.

EXAMPLE 5

Generation and Identification of Maize Transformants Containing the Modified Anthranilate Synthase Sequence

The anthranilate synthase sequence from pDPG602 was placed under control of the 35S CaMV promoter to determine if expression of this modified anthranilate synthase sequence was capable of conferring 5-MT or 6-MA resistance, and/or the tryptophan overproduction trait, to maize cell cultures. To generate the appropriate gene constructs with the 35S promoter, the plasmid pDPG165 was cleaved with restriction enzymes XbaI and KpnI, isolating the fragment containing the 35S CaMV promoter and the Tr7 terminator fused to the pUC19 plasmid backbone, and ligating this fragment to the 82 base pair XbaI/KpnI fragment from the multiple cloning site of the cloning vector pBSK to generate pDPG603.

pDPG602 was cleaved with restriction enzymes BamHI and Clal, which cleave sequences present in the multiple cloning sites flanking the cDNA fragment. The resultant 2107 base pair fragment was ligated into the BamHI and Clal sites present in the multiple cloning site of pDPG603 to generate a construct designated 35S/ASA2C28. The orientation of the recombinant plasmid obtained from this ligation is such that the modified anthranilate synthase cDNA sequence will be 3' to the 35S CaMV promoter and 5' to the Tr7 terminator sequence, thus placing the modified anthranilate synthase sequence under the transcriptional control of these two regulatory sequences such that a functional transcript encoding the modified anthranilate synthase enzyme would be produced in plant cells. A similar construct, using the wild type anthranilate synthase sequence from pDPG600, was prepared using the same strategy as that described for generation of 35S/ASA2C28. The wild-type construct, in which the wild type anthranilate synthase sequence is under control of the 35S and Tr7 sequences, was designated 35S/ASA2.

To evaluate expression of the anthranilate synthase sequences contained in 35S/ASA2C28 and 35S/ASA2 with respect to their ability to confer 5-MT or 6-MA resistance, or the trait of tryptophan overproduction, to maize

cells, these gene constructs were used in transformation experiments. These experiments involved co-transformation of either 35S/ASA2C28 or 35S/ASA2 along with the plasmid pDPG165 (35S/bar/Tr7), which allows for selection of transformants on bialaphos-containing media. Ears from the crosses

- 5 AB80(R1)xCW and CWxAB80(R1) were harvested 10 days post-pollination and the immature embryos were excised and plated on Medium 2365 (Medium 2366 with 2% sucrose, see Table 2) at 10 embryos per plate. Most of the embryos ranged in length from 1.2-1.7 mm with a few smaller than 1.2 mm. Embryos were arranged in concentric circles on the osmotic adjustment Medium 2366 (see
- 10 Table 2) at 30 embryos per plate 4 days after isolation.

Approximately four hours later, the embryos were bombarded using a helium gun (see Davis et al., PCT publication WO 95/06128, March 2, 1995). One of two plasmid combinations, 35S/ASA2+pDPG165 or 35S/ASA2C28+pDPG165, was used for each plate in order to obtain

- 15 transformants. After a 2-day recovery period, preselection was started on Medium 2377 (see Table 2) with 8-10 embryos per plate. After 15 days in medium 2377, the embryos were moved to selection media, either Medium 2066 (see Table 2) or Medium 2071, at one embryo per plate in order to eliminate cross-feeding between embryos. Transformants were identified after 12 weeks
- 20 of incubation on bialaphos selection media.

To identify those bialaphos-resistant transformants which carried either the 35S/ASA2C28 or 35S/ASA2 constructs, individual cultures were assayed by PCR analyses. DNA was extracted from 100-200 mg of callus tissue using the Puregene D-5500A Kit (Gentra Systems Inc.) and resuspended in 150 μ l of H₂O. Five μ l of each sample was then subjected to a PCR in a 50 μ l final volume containing 0.25 μ M of each primer A21-8 (SEQ. ID NO:10) and A21-9 (SEQ. ID NO:11), 3.0 mM MgCl₂, 20% glycerol, 200 μ M each dATP, dCTP, dGTP, and dTTP, 50 mM KCl, 10 mM Tris-HCl, pH 8.3, and 1.25U Taq DNA polymerase. Reactions were performed in the wells of microtiter plates in an MJ

- 25 Research thermal cycler (Model PTC-100) with a 96 well plate block. Thermal cycling conditions were as follows: initial denaturation at 97°C for 2 minutes; 3
- 30

cycles of 97°C for 1 minute, 56°C for 1 minute, and 72°C for 1 minute; 33 cycles of 94°C for 45 seconds, 56°C for 45 seconds, and 72°C for 45 seconds; and a final extension of 72°C for 4 minutes. Reaction products were analyzed by agarose gel electrophoresis for the presence of a 700 base pair product.

Table 2

| Media No. | Basal Medium | Sucrose | pH | Other Components (Amount/L) |
|-----------|--------------|---------|-----|---|
| 2366 | N6 | 12% | 5.8 | 1 mg 2,4-D 17 mg AgNO ₃ , 1 mg thiamineHCl, 0.5 mg nicotinic acid, 0.5 mg pyridoxine HCl, 2.9 g proline, 100 mg casamino acids, 2 mg glycine, 1.8g phytigel |
| 2377 | N6 | 2% | 5.8 | 1 mg 2,4-D, 1 mg bialaphos, 17 mg AgNO ₃ , 1 mg thiamine HCl, 0.5 mg nicotinic acid, 0.5 mg pyridoxine HCl, 2.9 g proline, 100 mg casamino acids, 2 mg glycine, 1.8 g phytigel |
| 2066 | N6 | 2% | 5.8 | 1.5 mg 2,4-D, 3 mg bialaphos, 1 mg thiamine HCl, 0.5 mg nicotinic acid, 0.5 mg pyridoxine HCl, 690 mg proline, 200 mg casamino acids, 2 mg glycine, 2.5 g phytigel |
| 10 | 2071 | N6 | 2% | 1 mg 2,4-D, 3 mg bialaphos, 1 mg thiamine HCl, 0.5 mg nicotinic acid, 0.5 mg pyridoxine HCl, 2.9 g proline, 100 mg, casamino acids, 2 mg glycine, 1.8 g phytigel |

| | | | | |
|-------|----|-----|-----|---|
| 211V | N6 | 2% | 5.8 | 1 mg 2,4-D, 17 mg AgNO ₃ , 1 mg thiamine HCl, 690 mg proline, 900 mg asparagine, 100 mg casamino acids, 500 mg MES |
| 211SV | N6 | 12% | 5.8 | 1 mg 2,4-D, 17 mg AgNO ₃ , 1 mg thiamine HCl, 690 mg proline, 900 mg asparagine, 100 mg casamino acids, 500 mg MES |
| 207D | N6 | 2% | 5.8 | 1.5 mg 2,4-D, 200 mg casamino acids, 3 mg bialaphos |

5 N6 medium is described in Chu et al., *Scientia Sinica*, 18, 659 (1975).

EXAMPLE 6

Analysis of Maize Transformants Containing the Gene Construct

35S/ASA2C28 for 5-methyltryptophan Resistance and for Tryptophan

10 Overproduction

A total of 15 transformants with 35S/ASA2C28 and pDPG165, or pDPG165 alone, that had been selected on bialaphos-containing medium, were evaluated for their ability to grow on 5-MT-supplemented media. Of these, 10 were PCR-positive for 35S/ASA2C28 and five contained only the pDPG165 construct. Of the 10 35S/ASA2C28-containing transformants, seven exhibited vigorous growth after four weeks on media supplemented with 5-MT at concentrations of 33 µM or 100 µM. Samples were scored for growth using an arbitrary scale, with a rating of "0" indicating little or no growth on 100 µM 5-MT media, a rating of "1" indicating some growth on 100 µM 5-MT media but not as much as on control media, and a rating of "2" indicating no difference in growth between control and 100 µM 5-MT media. The five controls exhibited little or no growth on 100 µM 5-MT media.

In another experiment, a total of 29 bialaphos-resistant transformants, 17 of which were PCR-positive for 35S/ASA2C28, 1 of which was PCR-positive for 35S/ASA2, and 11 of which contained neither construct, were transferred to 207D media supplemented with 100 μ M 5-MT, 200 μ M 5-MT, 100 μ M 6-MA, 5 or 200 μ M 6-MA. Five transformants carrying the 35S/ASA2C28 construct grew equally well on all of the media tested, four showed some inhibition of growth on either 100 μ M or 200 μ M 6-MA media but not on either concentration of the 5-MT media, and one showed reduced growth in the presence of both concentrations of 6-MA and 200 μ M 5-MT, but not on 100 μ M 5-MT. Another 10 eight lines exhibited little or no growth on either 5-MT or 6-MA media, suggesting that the transgene was not expressed in those lines. Most of the untransformed lines exhibited little or no growth on either 6-MA or 5-MT media, and the single 35S/ASA2 transformant tested did not grow on either type of supplemented media. Thus, it appears that 6-MA is a more potent inhibitor of 15 growth than is 5-MT at equimolar concentrations. These data indicate that cells transformed with the 35S/ASA2C28 construct express the 5-Mtr trait and can exhibit vigorous growth at concentrations of 5-MT or 6-MA up to 200 μ M.

To determine if the presence of the 35S/ASA2C28 construct in these transformants is associated with tryptophan overproduction, the levels of free 20 tryptophan in these callus cultures were evaluated using a modification of the procedure described by Jones et al. (*Analyst*, 106, 968 (1981)). A weighed sample of callus tissue was ground with a Polytron (Brinkmann Instruments) for 1 minute in extraction buffer (25% acetonitrile, 10 mM sodium acetate buffer, pH 7.0) at a ratio of 5 ml buffer to 1 gram of tissue. Samples were shaken at 25 about 4°C for 2 hours and then centrifuged at 3500 x g for 10 minutes followed by filtration through a 0.5 micron filter. The resultant supernatant was analyzed by HPLC using a reverse phase, C-18 column (4.5 x 25 cm), fluorescence detection (excitation 254 nm, emission 320 nm) and isocratic elution (25 mM sodium acetate buffer, pH 3.8:methanol, 3:1 vol/vol) at a flow rate of 1 30 ml/minute and a temperature of 50°C. Twenty μ l of sample was injected for

each run. Analysis of peaks was performed using MAXIMA software (Waters Instruments) with further data processing in an EXCEL (Microsoft) spreadsheet.

Results of tryptophan analyses of these lines are shown in Table 3 and Table 4. Of the lines assayed, two (TRPA030 and TRPB044) contained

5 substantially elevated levels of tryptophan relative to controls. These levels were in the range of 60 to 102 ppm free tryptophan. Most of the other 35S/ASA2C28 lines contained levels in the range of 20 to 30 ppm free tryptophan, while levels in most of the controls were 6 to 11 ppm. The level of tryptophan in line TRPA030 is similar to that of the original C28 callus line that is resistant to 10 5-MT. These data indicate that maize cells containing the gene construct 35S/ASA2C28 are capable of tryptophan overproduction in comparison to maize cells that do not contain this construct.

Table 3

| | TRANSFORMANT | 35S/ASA2 | Trp (ppm) | Growth Rating |
|----|--------------|----------|-----------|---------------|
| | | C28 | | |
| 15 | TRPA 030 | + | 101.6 | 2 |
| | TRPA 015 | + | 28.8 | 2 |
| | TRPA 032 | + | 27.0 | 1 |
| | TRPA 027 | + | 26.3 | 2 |
| | TRPA 016 | + | 16.8 | 2 |
| 20 | TRPA 011 | + | 14.7 | 2 |
| | TRPA 033 | + | 14.4 | 1 |
| | TRPA 013 | - | 15.6 | 0 |
| | TRPA 019 | - | 10.1 | 0 |

Table 4

| Transformant | Transgene | 207D | +100 μ M 5-MT | +200 μ M 5-MT | +100 μ M 6-MA | +200 μ M 6-MA | TRP (ppm) |
|--------------|-----------|------|-------------------|-------------------|-------------------|-------------------|-----------|
| TRPB 044 | p605 | 2 | 2 | 2 | 2 | 2 | 62.5 |
| TRPB 043 | p605 | 2 | 2 | 2 | 2 | 2 | 25.3 |
| TRPB 048 | p605 | 2 | 2 | 2 | 2 | 2 | 24.98 |
| TRPB 049 | p605 | 2 | 2 | 2 | 2 | 2 | 22.41 |
| TRPB 019 | p605 | 2 | 2 | 1 | 1 | 1 | 20.7 |
| TRPB 042 | p605 | 2 | 2 | 2 | 2 | 2 | 20.7 |
| TRPB 051 | p605 | 2 | 2 | 2 | 1 | 1 | 15.02 |
| TRPB 011 | p605 | 2 | 2 | 2 | 2 | 1 | 12.56 |
| TRPB 050 | p605 | 2 | 2 | 2 | 1 | 1 | 10.49 |
| TRPB 017 | p605 | 2 | 1 | 0 | 0 | 0 | 0 |
| TRPB 022 | p605 | 2 | 0 | 0 | 0 | 0 | 0 |
| TRPB 025 | p605 | 2 | 0 | 0 | 0 | 0 | 0 |
| TRPB 031 | p605 | 2 | 2 | 1 | 1 | 0 | 0 |
| TRPB 038 | p605 | 2 | 0 | 0 | 0 | 0 | 0 |
| TRPB 040 | p605 | 2 | 0 | 0 | 0 | 0 | 0 |
| TRPB 046 | p605 | 2 | 1 | 1 | 1 | 0 | 0 |
| TRPB 021 | p605 | 2 | 2 | 1 | 1 | 0 | 0 |
| TRPB 033 | p604 | 2 | 0 | 0 | 0 | 0 | 6.31 |
| TRPB 029 | --- | 2 | 2 | 2 | 1 | 1 | 21.68 |
| TRPB 014 | --- | 2 | 0 | 0 | 0 | 0 | 11.07 |
| TRPB 037 | --- | 2 | 0 | 0 | 0 | 0 | 10.79 |
| TRPB 028 | --- | 2 | 1 | 1 | 1 | 1 | 10.42 |
| TRPB 020 | --- | 2 | 0 | 0 | 0 | 0 | 7.77 |
| TRPB 015 | --- | 2 | 1 | 0 | 0 | 0 | 0 |
| TRPB 018 | --- | 2 | 0 | 0 | 0 | 0 | 0 |
| TRPB 024 | --- | 2 | 1 | 1 | 1 | 0 | 0 |
| TRPB 039 | --- | 2 | 1 | 0 | 0 | 0 | 0 |
| TRPB 041 | --- | 2 | 0 | 0 | 0 | 0 | 0 |
| TRPB 047 | --- | 2 | 1 | 0 | 0 | 0 | 0 |

EXAMPLE 7

Analysis of Maize Transformants Containing the Gene Construct
35S/ASA2C28 for Anthranilate Synthase Activity Exhibiting Reduced
Sensitivity to Tryptophan Feedback Inhibition

5 To determine whether maize transformants carrying the 35S/ASA2C28 gene construct contained anthranilate synthase activity which was less sensitive to tryptophan feedback inhibition than the anthranilate synthase present in control cells, anthranilate synthase enzyme assays were

10 performed essentially as described by Widholm (*Biochimica et Biophysica Acta*, 279, 48 (1972)). Five grams of callus tissue was ground with a Polytron (Brinkmann Instruments) for 1 minute with an equal volume of extraction buffer (100 mM HEPES, pH 7.5, 0.4 mM β -mercaptoethanol, 0.2 mM EDTA, 1 mM MgSO₄, 100 μ M glutamine, 40% glycerol). The resultant homogenate was

15 centrifuged at 6000 x g for 20 minutes and the supernatant was de-salted on a Biorad P6-DS column (12 ml bed volume, 4 ml sample applied). The enzyme assay was run at 30°C and the production of anthranilate was continuously measured spectrofluorometrically at a 340 nm excitation wavelength and a 400 nm emission wavelength (slit widths 5 and 10 nm, respectively). The final 1 ml

20 reaction mixture contained up to 250 μ l of enzyme extract and 8 μ moles of MgSO₄, 40 μ moles of L-glutamine and 1 μ mole of chorismate. Reactions were run in the presence of 0 to 15 μ M tryptophan. Protein content of samples was determined by the standard Bradford assay. Anthranilate standards were used to quantify emission readings.

25 As can be seen in Figure 4, the cell lines TRPA030 and TRPA015, both of which contain the 35S/ASA2C28 gene construct, exhibited ability to grow on 100 μ M 5-MT, contained higher tryptophan levels relative to tryptophan levels in controls, and exhibited levels of anthranilate synthase activity higher than those of the control cell line TRPA013, which does not

30 contain the 35S/ASA2C28 construct. The anthranilate synthase activity in the

35S/ASA2C28 lines also exhibited greater tolerance to tryptophan inhibition than did the control.

EXAMPLE 8

Characterization of the Transcriptional Promoter Region from the Maize

5 ASA2 Gene

To evaluate the utility of the 5' regulatory sequence of the maize anthranilate synthase gene ASA2 in directing gene expression in maize cells, a fragment from the clone pDPG668 was inserted 5' to a GUS reporter gene. This was done by restriction mapping pDPG668, which allowed the identification of 10 an Xba/PvuII fragment that contains 12 base pairs of 5'UTR (including the PvuII site) and about 1100 base pairs of 5' flanking sequence. The Xba/PvuII fragment from pDPG668 was isolated, the Xba overhang filled in with the Klenow fragment of DNA polymerase, and the resultant blunt-ended fragment was substituted for the 35S promoter in pDPG126 (35S/GUS/TR7) by cleaving 15 that plasmid with BamH1 and HindIII to remove the 35S promoter fragment, filling in with Klenow fragment of DNA polymerase I to generate blunt ends, treating with alkaline phosphatase, then ligating this linear vector to the pDPG668 blunt end fragment to generate pDPG671 (Asa2/GUS/Tr7). The pDPG671 construct was introduced into immature maize embryos by particle 20 bombardment as follows: embryos were excised at 10 days post-pollination, maintained on 211V (Table 2) solid medium for two days at 27°C, then transferred to 211SV (Table 2) medium four hours prior to bombardment (see Davis et al., PCT publication WO 95/06128, March 2, 1995). Embryos remained on 211SV for two days post-bombardment, at which time they were 25 transferred to histochemical GUS assay buffer (0.1 M sodium phosphate, pH 7.0, 5 mM potassium ferricyanide, 1 mM EDTA, 1% Triton X-100, 0.3% X-glucuronide). A limited number of blue-staining spots was observed after 24 hours of staining, indicating that the Xba/PvuII fragment from pDPG668 was capable of directing expression of the GUS reporter gene in maize cells. The 30 insert was subjected to nucleotide sequencing analysis. The 1200 nucleotides 5' to the putative translational start site are shown in Figure 5 (SEQ ID NO:14).

EXAMPLE 9

Procedures that Facilitate Identification of Plants Carrying Either the ASA2 C28 Allele or for Plants Expressing the Transgene Present in the 35S/ASA2C28 Gene Construct

5 As indicated above, the biological nature of 5-MT resistance in the proprietary C28 maize line is due to a mutation in a gene encoding the enzyme anthranilate synthase. The mutation results in a reduction in sensitivity of the enzyme to tryptophan feedback inhibition, therefore allowing for tryptophan overproduction in the cell. This modified enzyme could exhibit a
10 similar tolerance to other tryptophan analogues that exert phytotoxic effects via the tryptophan pathway. The use of one such analogue, 6-MA, was evaluated in assays designed to distinguish plants carrying the maize ASA2 C28 allele from those containing only wild type alleles at this locus.

 Spray trials were conducted in which 7-to-10 day-old seedlings of
15 C28/C28 and wild-type sources were sprayed with a solution of 10 mM 6-MA, 10% glycerol, 0.5% Tween-20. Five days after spraying, the wild type plants exhibited chlorosis, particularly between leaf veins, while the C28 plants appeared normal. This indicated that this 6-MA formulation was toxic to wild-type plants but not to C28/C28 plants. The effects of 5-MT and 6-MA on wild
20 type and C28 plants was then compared. This experiment consisted of treating plants representing a variety of genetic backgrounds in which the C28 allele was segregating in a 1:1 ratio. Rows of 20 kernels each, representing eight different C28 backcross conversion populations, were planted in two identical flats. One flat was sprayed with 5-MT and the other with 6-MA (both at a concentration of
25 10 mM in a solution containing 10% glycerol and 0.5% Tween-20). Plants were sprayed 8 days after planting. Spray treatments were evaluated both 6 and 10 days after spraying. In all cases, clear segregation for resistance to both 5-MT and 6-MA were apparent, and in some cases the effects were more dramatic than in others. In all cases, the 6-MA treatment resulted in a much clearer separation
30 of resistant and sensitive plants.

Given the dramatic effects of 6-MA on sprayed seedlings, it was decided to evaluate the germination ability of seeds carrying the C28 allele in the presence of 6-MA. Previous work with 5-MT, as described in U.S. Patent No. 4,581,847, indicated that 5-MT inhibited germination in excised embryos. Seeds 5 from seven segregating C28 backcross populations, in addition to C28/C28 and wild type control sources, were germinated between filter paper saturated with a solution of 100 μ M 6-MA. After six days incubation, individual seedlings were scored for length of the primary root. All of the C28/C28 seedlings exhibited roots which were greater than 8 cm in length, and root length in all of the control 10 wild type sources was less than 3 cm. In those samples segregating for the C28 allele, plants scored as sensitive were those with roots less than 5 cm in length, and those scored as positive had roots greater than 7 cm in length. This experiment clearly demonstrates that 6-MA at a concentration of 100 μ M can be used to identify plants carrying the C28 allele of ASA2 in a seed germination 15 assay.

The reliability of both the seedling spray assay and the seed germination assay involving 6-MA treatment for the identification of plants carrying the ASA2 C28 allele was verified through the use of a molecular marker which physically defines the C28 allele of the ASA2 gene. As described above, 20 the modification in the C28 allele involves a T to A substitution at position 1194 in the pDPG600 nucleotide sequence. This substitution occurs in the context CATG, which is the recognition sequence for the restriction enzyme NlaIII. The T to A substitution present in the ASA2 C28 allele results in a change of sequence from CATG to CAAG, and therefore also results in the loss of the 25 NlaIII recognition sequence at this position. The absence of this site therefore serves as a diagnostic molecular marker for the C28 ASA2 allele. Thus, by amplifying a 500 base pair fragment in a PCR using primers A21-15 (SEQ ID NO:13) and A21-16 (SEQ ID NO:15), which flank this site, and digesting the product with NlaIII, the uncleaved product (500 base pairs) from the C28 allele 30 is easily distinguished from the cleavage products of wild type alleles (400 + 100 base pair) by agarose gel electrophoresis. This marker has been used to identify

the presence of the C28 allele in individuals from populations segregating both 6-MA and 5-MT resistance.

EXAMPLE 10

Analysis of Transgenic Plants Transformed with 35S/ASA2C28

5 Transgenic plants were regenerated from callus cultures containing 35S/ASA2C28. These plants were used as male or female parents in crosses with plants of elite inbred lines. Kernel progeny from these plants were evaluated for their ability to germinate in the presence of 6-MA and for grain tryptophan content by employing methods described above (Example 9 and

10 Example 6, respectively). Since the transformation process is expected to generate plants hemizygous for a transgene, the kernel progeny in which a transgenic plant is used as one of the parents are expected to segregate 1:1 for the presence of the transgene: half of the progeny will be hemizygous for the transgene and half will be wildtype. The results of an assay in which the ability

15 of kernel progeny from several 35S/ASA2C28 transformants to germinate in the presence of 6-MA was evaluated are shown in Table 5. Progeny from three of these transformants, including TRPA 030, exhibited segregation ratios for 6-MA resistance at ratios close to 1:1. Three other transformants, which exhibited ability to grow on 6-MA media as callus cultures (Table 4), did not produce

20 kernel progeny which were capable of germinating in the presence of 6-MA. This is likely due to differences in the level of expression of the 35S/ASA2C28 transgene in callus cultures and in seed of these specific transformants. These data indicate that transformant TRPA 030, which contains exceptionally high levels of tryptophan in callus cultures (Table 3), exhibits the ability to germinate

25 in the presence of 6-MA.

Table 5

| Female parent | Male parent | Number | Number | Root length |
|---------------|-------------|--------|--------|-------------|
| TRPA 030 | CV | 4 | 6 | 21cm |
| TRPB 049 | AW | 3 | 7 | 10cm |
| AW | TRPB 050 | 5 | 5 | 22cm |
| TRPA 016 | AW | 0 | 10 | <3cm |
| TRPB 042 03 | AW | 0 | 10 | <3cm |
| Z.Y | TRPB 043 | 0 | 10 | <3cm |

To evaluate the effect of 35S/ASA2C28 transgene expression on tryptophan overproduction in the grain, tryptophan levels in individual kernels from an ear in which transformant TRPA 030 was used as a female parent were determined. As a control, tryptophan levels in kernel progeny from an ear in 5 which transformant TRPB 039, which carries only the selectable marker bar transgene, and not the 35S/ASA2C28 transgene, was used as a female parent. None of the TRPB 039 kernel progeny contained tryptophan levels over 22.8 ppm (range 16.0 to 22.8 ppm), while half of the TRPA 030 progeny exhibited 10 tryptophan levels in the 62-98.9 ppm range and the other half exhibited levels in the 16.5-30.4 ppm range (Table 6). These data clearly demonstrate that kernel progeny resulting from crosses in which TRPA 030 is used as a parent contain levels of tryptophan that are significantly higher than those of transgenic plants that do not carry the 35S/ASA2C28 transgene.

Table 6

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| Sample | Trp | EAR NAME |
|--------|------|---------------|
| 1 | 19.5 | TRPB 039 X CV |
| 2 | 19.2 | TRPB 039 X CV |
| 3 | 21.7 | TRPB 039 X CV |
| 4 | 17.8 | TRPB 039 X CV |
| 5 | 17.2 | TRPB 039 X CV |
| 6 | 20.6 | TRPB 039 X CV |
| 7 | 21.1 | TRPB 039 X CV |
| 8 | 17.6 | TRPB 039 X CV |
| 9 | 19.3 | TRPB 039 X CV |
| 10 | 22.2 | TRPB 039 X CV |
| 11 | 16.0 | TRPB 039 X CV |
| 12 | 18.7 | TRPB 039 X CV |
| 13 | 16.6 | TRPB 039 X CV |
| 14 | 16.9 | TRPB 039 X CV |
| 15 | 22.8 | TRPB 039 X CV |
| 16 | 21.6 | TRPB 039 X CV |
| 17 | 51.4 | TRPA 030 X CV |
| 18 | 21.2 | TRPA 030 X CV |
| 19 | 62.4 | TRPA 030 X CV |
| 20 | 63.1 | TRPA 030 X CV |
| 21 | 72.9 | TRPA 030 X CV |
| 22 | 98.9 | TRPA 030 X CV |
| 23 | 65.3 | TRPA 030 X CV |
| 24 | 18.1 | TRPA 030 X CV |
| 25 | 62.0 | TRPA 030 X CV |
| 26 | 71.0 | TRPA 030 X CV |
| 27 | 30.4 | TRPA 030 X CV |
| 28 | 18.8 | TRPA 030 X CV |
| 29 | 22.4 | TRPA 030 X CV |
| 30 | 16.5 | TRPA 030 X CV |
| 31 | 25.7 | TRPA 030 X CV |
| 32 | 27.3 | TRPA 030 X CV |

EXAMPLE 11

Use of 35S/ASA2C28 as a selectable marker for plant transformation

Since the 35S/ASA2C28 construct was found to confer resistance to 5-MT or 6-MA in maize cells (Example 6), the usefulness of this construct as a selectable marker was evaluated in a series of transformation experiments. These experiments were conducted in a manner similar to those described in Example 5 above in that immature maize embryos were bombarded with either plasmid combination 35S/ASA2C28 + pDPG165 or combination 35S/ASA2+pDPG165. However, rather than selecting for transformants on bialaphos-containing media, the selection process was performed on media containing 6-MA, as follows: two days post-bombardment, preselection was initiated with 8-10 embryos per plate on Medium 211VW (Medium 211V from Table 2 supplemented with 6-MA to 33 uM). After 15 days on Medium 211VW, the embryos were transferred to the selection Medium 211X (Medium 211V minus silver nitrate and supplemented with 6-MA to 100 uM) at one embryo per plate. Transformants bombarded with the 35S/ASA2C28+pDPG165 combination were identified after 12 weeks of selection on the 100 uM 6-MA selection medium. No transformants were obtained from embryos bombarded with the 35S/ASA2+pDPG165 combination. The number of transformants obtained with the 35S/ASA2C28+pDPG165 combination by way of 6-MA selection is similar to that obtained by way of bialaphos selection. This clearly demonstrates that use of the 35S/ASA2C28 construct and 6-MA selection allows for identification of transformants in a manner similar to that in which pDPG165 and bialaphos selection is used for identification of transformants.

All publications and patents are incorporated by reference herein, as though individually incorporated by reference. The invention is not limited to the exact details shown and described, for it should be understood that many variations and modifications may be made while remaining within the spirit and scope of the invention defined by the claims.

WHAT IS CLAIMED IS:

1. An isolated and purified DNA molecule comprising a first DNA segment encoding an anthranilate synthase substantially resistant to inhibition by free L-tryptophan or an amino acid analog of tryptophan.
2. The DNA molecule of claim 1 wherein the first DNA segment encodes a plant anthranilate synthase.
3. The DNA molecule of claim 2 wherein the first DNA segment encodes a maize anthranilate synthase.
4. The DNA molecule of claim 3 wherein the first DNA segment encodes a protein having SEQ ID NO:16.
5. The DNA molecule of claim 3 wherein the first DNA segment consists essentially of SEQ ID NO:12.
6. An isolated and purified DNA molecule comprising a first DNA segment encoding a maize anthranilate synthase.
7. The DNA molecule of claim 6 wherein the first DNA segment encodes a protein having SEQ ID NO:2.
8. The DNA molecule of claim 6 wherein the first DNA segment consists essentially of SEQ ID NO:1.
9. The DNA molecule of claims 1, 2, 3, 4, 5, 6, 7 or 8 further comprising a second DNA segment encoding an amino terminal chloroplast transit peptide, which is operably linked to the first DNA segment.

10. A method of imparting tolerance to an amino acid analog of tryptophan to a plant cell comprising:
 - (a) introducing an expression cassette comprising a first DNA segment encoding an exogenous anthranilate synthase operably linked to a promoter functional in a plant cell into cells of a susceptible plant to yield transformed plant cells, wherein the anthranilate synthase is substantially resistant to inhibition by free L-tryptophan or an amino acid analog of tryptophan; and
 - (b) expressing the anthranilate synthase encoded by the first DNA segment in the plant cells so as to yield an amount of said anthranilate synthase effective to render the transformed plant cells substantially tolerant to an amount of free L-tryptophan or an amino acid analog of tryptophan that inhibits the growth of the untransformed cells of the susceptible plant.
11. The method according to claim 10 wherein the expression of the first DNA segment is inducible.
12. The method according to claim 10 wherein the expression cassette further comprises plasmid DNA.
13. The method according to claim 10 wherein the first DNA segment encoding the anthranilate synthase is introduced into plant cells by a method selected from the group consisting of electroporation, microinjection, protoplast transformation, microprojectile bombardment, *Agrobacterium*-mediated transformation, and liposomal encapsulation.
14. The method according to claim 10 wherein the anthranilate synthase is a plant anthranilate synthase.

15. The method according to claim 14 wherein the plant anthranilate synthase is a maize anthranilate synthase.
16. The method according to claim 15 wherein the maize anthranilate synthase has SEQ ID NO:16.
17. The method according to claim 10 wherein the first DNA segment consists essentially of SEQ ID NO:12.
18. The method according to claim 10 wherein the plant cells comprise cells of callus, embryos, meristematic tissue, gametic tissue, or cultured cells in suspension.
19. The method according to claim 10 wherein the amino acid analog is 5-methyltryptophan.
20. The method according to claim 10 wherein the expression cassette further comprises a second DNA segment encoding an amino terminal chloroplast transit peptide which is operably linked to the first DNA segment.
21. The method according to claim 10 wherein the amino acid analog is 6-methylanthranilate.
22. The method according to claim 8 wherein the anthranilate synthase encoded by the first DNA segment is expressed in chloroplasts of the plant cells.
23. A transformed plant regenerated from the transformed plant cells obtained by the method of claim 10.

24. A transformed seed of the transformed plant of claim 23.
25. A method for altering the tryptophan content in a plant comprising:
 - (a) introducing into the cells of a susceptible plant an expression cassette comprising a recombinant DNA segment encoding an anthranilate synthase operably linked to a promoter functional in a plant cell to yield transformed plant cells, wherein the DNA segment encodes an anthranilate synthase which is substantially resistant to inhibition by free L-tryptophan or an amino acid analog of tryptophan; and
 - (b) regenerating a differentiated plant from said transformed plant cells wherein the cells of the differentiated plant express the anthranilate synthase encoded by the DNA segment in an amount effective to increase the tryptophan content in the cells of the differentiated plant relative to the tryptophan content in the cells of the untransformed susceptible plant.
26. The method according to claim 25 wherein the tryptophan content of the transformed plant cells is about 1.1- to 50-fold higher than that of the tryptophan content of the susceptible plant cells of step (a).
27. A method of producing anthranilate synthase comprising:
 - (a) introducing an expression cassette comprising a DNA segment encoding an anthranilate synthase into a population of cells;
 - (b) expressing the DNA segment encoding the anthranilate synthase in said cells so as to yield anthranilate synthase; and
 - (c) recovering the anthranilate synthase from said cells.

28. An expression cassette comprising a first DNA segment encoding an anthranilate synthase which is substantially resistant to inhibition by free L-tryptophan or an amino acid analog of tryptophan, wherein the first DNA segment is joined in correct reading frame at its 5'-terminus to a second DNA segment encoding an amino-terminal chloroplast transit peptide, and wherein the first DNA segment and the second DNA segment are under the transcriptional and translational regulatory control of regulatory regions functional in a plant cell.
29. The expression cassette of claim 28 which further comprises a selectable marker gene or a reporter gene.
30. An isolated and purified DNA molecule comprising a DNA segment encoding transcription regulatory elements for a plant anthranilate synthase.
31. The DNA molecule of claim 30 wherein the plant anthranilate synthase is a maize anthranilate synthase.
32. The DNA molecule of claim 30 wherein the DNA segment consists of SEQ ID NO:14.
33. A method of selecting transformed plant cells, comprising:
 - (a) introducing into a plant cell an expression cassette comprising a first DNA segment encoding an anthranilate synthase which is substantially resistant to inhibition by free L-tryptophan or an amino acid analog of tryptophan to yield a transformed plant cell; and
 - (b) culturing the transformed plant cell in an amount of free L-tryptophan or an amino acid analog of tryptophan that

inhibits the growth of a plant cell which does not contain the first DNA segment.

34. An isolated and purified DNA molecule comprising a DNA segment encoding a beta subunit of an anthranilate synthase.
35. The DNA molecule of claim 34 wherein the DNA segment encodes a beta subunit of a plant anthranilate synthase.
36. The DNA molecule of claim 35 wherein the DNA segment encodes a beta subunit of a maize anthranilate synthase.
37. The DNA molecule of claim 36 wherein the DNA segment consists essentially of SEQ ID NO:8.

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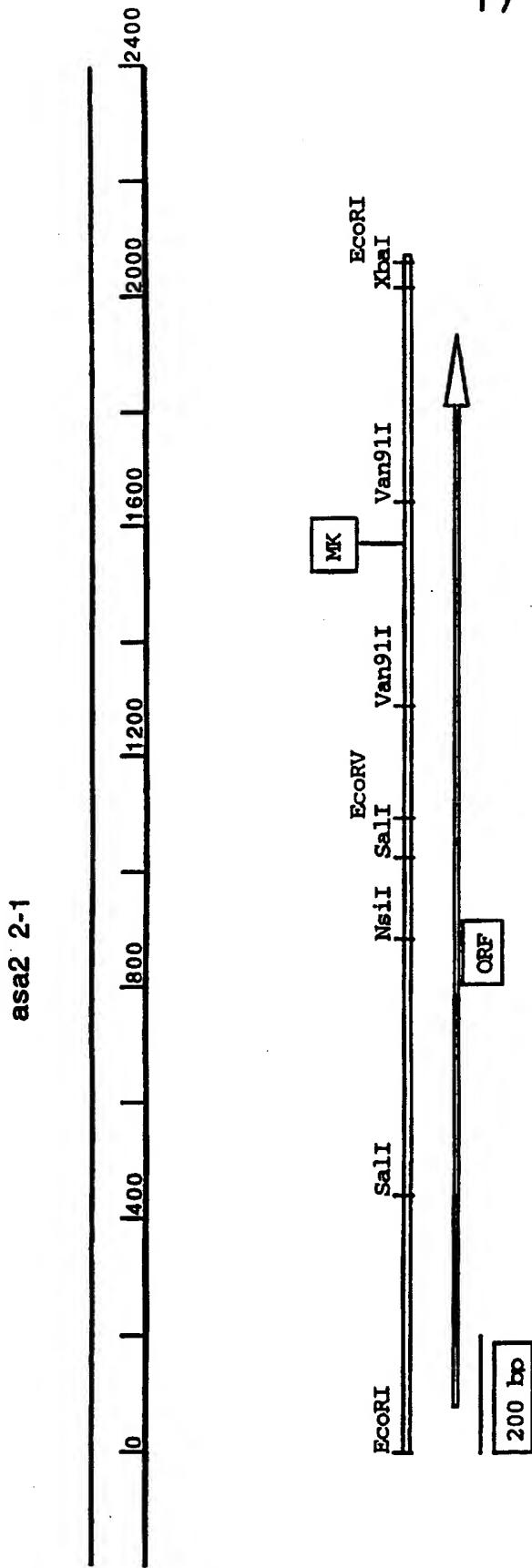


FIGURE 1

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FIGURE 2

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Alignment of maize and *arabidopsis* Anthranilate Synthase alpha subunit sequences

| | | | |
|-----|---|-----|--|
| 90 | RELLAETHVAPSRVIVEYARIILVRAGTVFTRRTSISLSCGSGVKC&AAVIFQASPVISRSAMAIAKAABEDGRPFEEAARRGSGKGNLUP S5- S5- S5-- | 180 | YVREKEDDQKTTTPEKSOV S-S S-S S-S |
| 91 | ATASA2 ATASA2 ATASA1 | 174 | YVREKEDDQKTTTPEKSOV S-S S-S S-S |
| 92 | ATASA2 ATASA2 ATASA1 | 159 | YVREKEDDQKTTTPEKSOV S-S S-S S-S |
| 93 | ATASA2 ATASA2 ATASA1 | 268 | YVREKEDDQKTTTPEKSOV S-S S-S S-S |
| 94 | ATASA2 ATASA2 ATASA1 | 249 | YVREKEDDQKTTTPEKSOV S-S S-S S-S |
| 95 | ATASA2 ATASA2 ATASA1 | 358 | YVREKEDDQKTTTPEKSOV S-S S-S S-S |
| 96 | ATASA2 ATASA2 ATASA1 | 352 | YVREKEDDQKTTTPEKSOV S-S S-S S-S |
| 97 | ATASA2 ATASA2 ATASA1 | 339 | YVREKEDDQKTTTPEKSOV S-S S-S S-S |
| 98 | ATASA2 ATASA2 ATASA1 | 448 | YVREKEDDQKTTTPEKSOV S-S S-S S-S |
| 99 | ATASA2 ATASA2 ATASA1 | 442 | YVREKEDDQKTTTPEKSOV S-S S-S S-S |
| 100 | ATASA2 ATASA2 ATASA1 | 429 | YVREKEDDQKTTTPEKSOV S-S S-S S-S |
| 101 | ATASA2 ATASA2 ATASA1 | 537 | YVREKEDDQKTTTPEKSOV S-S S-S S-S |
| 102 | ATASA2 ATASA2 ATASA1 | 531 | YVREKEDDQKTTTPEKSOV S-S S-S S-S |
| 103 | ATASA2 ATASA2 ATASA1 | 518 | YVREKEDDQKTTTPEKSOV S-S S-S S-S |
| 104 | ATASA2 ATASA2 ATASA1 | 615 | YVREKEDDQKTTTPEKSOV S-S S-S S-S |
| 105 | ATASA2 ATASA2 ATASA1 | 621 | YVREKEDDQKTTTPEKSOV S-S S-S S-S |

FIGURE 3

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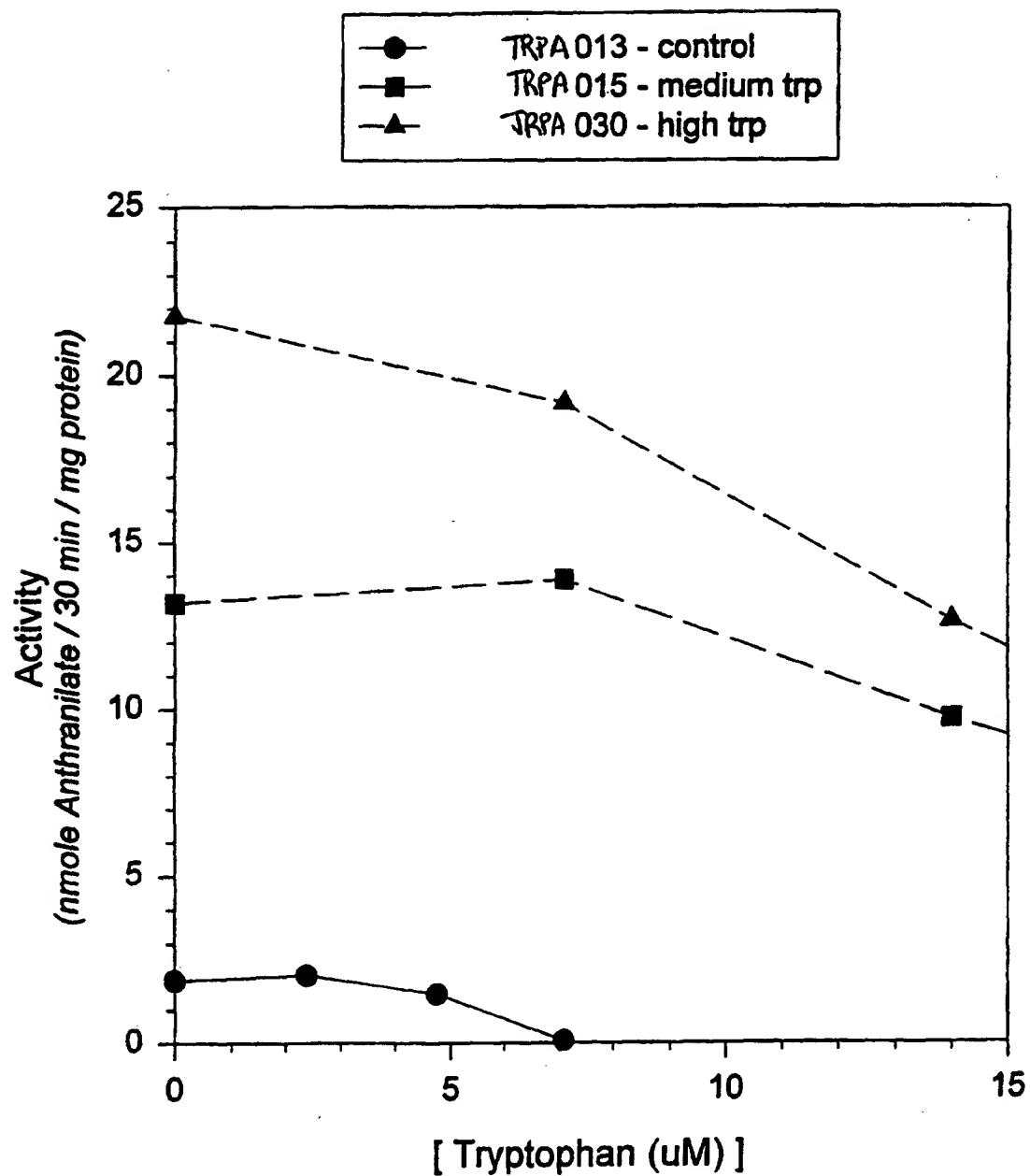


FIGURE 4

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| | |
|---|------|
| TCTAGATATTCTTGTGGTGTGTTACTAGCTAATTCTTAATCTATCTTTAACAGTCCCCAC | 75 |
| CCACCCACACGAGCACAACTTCACTGTGGCTCCATGGCTGCAACGGCCACGGTGGCTTGG | 150 |
| TGTGGGACCGACACCGGGATTTCGCTGCAACAACTGGCTGCAAGACTGTCAGCTTCAATTGGCCCTGTCTTACTGTCTTGG | 225 |
| TACGATAAGGTACCCGCAAGGGTGTGCAAGACTGTAACCTCTGTGTCAGTGTCCAAAATAAGAATTATT | 300 |
| TTTCACAAATTCTCACTGTAAATCTTCAATGAAAGTTGTAGGAATTCAATCCACAAACATAATAACAA | 375 |
| CATTATTACTATATATTAGTTGATTATTGAGATGAAAGAGTAATTATTATATTATTTGACAAGTACTG | 450 |
| TCTCTGTCAAGGCTGGCTCCAGGCAAGGTTCGGCAAAGCTTCCGTACCCCTAAATAGAGAATCAAACATCTCA | 525 |
| TGCTCTAGGAGCATTTCTGAACGGTCCTCTAAATTAGTGAACGCTGGATTCTTATATAAGAATTTC | 600 |
| TCTAAATGATCTCTATCTTACCTTTAAATAATCGGTTAGCAAAACCTAAATATGTATATACTATT | 675 |
| GAGGGTATTGATAATACATAGTTAAATAAAATAATCTTTAAATAATTTAGTGTATTAGCTTAA | 750 |
| GACCTGATTAAAGGACGCTGGTGGAGAGAAAGTAGATAAAAGGATTAAGAGATAAGAA | 825 |
| AGATATAATAATGATGATAATTACATTGATTGAGACAGGCTCACCCATCATCATCAATCAGGAAAGGAGCTTAA | 900 |
| TCTTCCTCCCTTCACCGTACCGAGACCCAAACCAAGGCTGGCAACTCTTAACTCTTAACTCT | 975 |
| TCTCCATTAAATCTGGTTCTCCTTGGAAATCTGCTGGCAAGTCCCAGGACCCGAATGCCCCACTCTTATTACCT | 1050 |
| GCTAATTGTAATTCTCTTCTCCTAATCGGGTTGGAGCTG | 1084 |

FIGURE 5

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| | |
|---|-----|
| TTGCCAAACC CTTTTACTGC TGCGAGGTAC CACAGCTTGG TCATTGAGCA | 50 |
| AGAAACCTTC CCACATGATG CTTGGAGGC TACTGCATGG ACTGAAGATG | 100 |
| <u>GACTTATCAT GGCTGCTCGC CACAAGAATA CAAACACATC CAGGGTGTCC</u> | 150 |
| <u>AATTCCACCC GGAGAGCA</u> | 168 |

FIGURE 6

FIGURE 7

| | |
|--|-----|
| MESLAATSVF APSRVAVPAA RALVRAGTVV PTRRTSSRSG TSGVXCSAAV | 50 |
| TPOASFVISR SAAAAKAAEE DKRRFFEARAA RGSGKGNLVP MWECIKGNLV | 100 |
| PMWECIVSDR LTFVLAYRCL VPEDNVDAPS FLPHSVBQGP QGTTNVGRYS | 150 |
| MVGAHPVMEI VAJDHKVTIM DHEKSQVTEQ VVDDDFMQIPR TMMEGWHPQQ | 200 |
| IDEPLPESFSG GWVGFPBYDT VRYVEIOCKLP FSSAPQODRN LPDVHLGLYD | 250 |
| DVLVFDNEVK XUVVIEENVNV DREHASVHEAY QDGRSPRLML LSXVHNHNP | 300 |
| TLSPGPFVNLH TRKFUTPLNK STMTSDEYKN AVLQAKEHIM AGDIPQIVLS | 350 |
| QRFERRTYAN PFEVYRALRI VNPSPYKAYV QARGCVLVAS SPEILTRVSK | 400 |
| GKIINRPLAG TVRRGKTEKE DQMQEQQLLS DEKQCAEHIM LVDLGRMDVG | 450 |
| XVSKPGGSVK VHELIIRYS HVMHISSTVS GOLDDDEHQSW DALRAALPVG | 500 |
| TVSGAPKVKA MELIDKLEVT RRGPYSGGLG GISFDGDMQI ALSLRTIVFS | 550 |
| TAPSHENTMYS YKDADRRREW VAHLQAGAGI VADSSFDDEQ RECENTAAAL | 600 |
| ARAAIDLAEWA FVNKE | 615 |

INTERNATIONAL SEARCH REPORT

Application No

P. /US 97/00983

A. CLASSIFICATION OF SUBJECT MATTER

| | | | | | |
|-------|-----------|-----------|----------|----------|----------|
| IPC 6 | C12N15/82 | C12N15/60 | C12N1/14 | C12N9/88 | A01H1/00 |
| | A01H4/00 | | | | |

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 C12N A01H

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

| Category * | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|------------|---|--|
| X | EP 0 174 791 A (MOLECULAR GENETICS RESEARCH &) 19 March 1986 see the whole document --- | 1-3,6, 10, 12-15, 18,19, 23-26 |
| X | PLANT PHYSIOLOGY, vol. 110, January 1996, pages 51-59, XP002032320 LI, J. AND LAST, R.L.: "THE ARABIDOPSIS THALIANA trp5 MUTANT HAS A FEEDBACK-RESISTANT ANTHRANILATE SYNTHASE AND ELEVATED SOLUBLE TRYPTOPHAN" see the whole document --- | 1,2,10, 12-14, 18-26,33 -/- |

Further documents are listed in the continuation of box C.

Patent family members are listed in annex.

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1

Date of the actual completion of the international search

4 June 1997

Date of mailing of the international search report

12.06.97

Name and mailing address of the ISA

European Patent Office, P.B. 5818 Patentstaan 2
NL - 2280 HV Rijswijk
Tel. (+31-70) 340-2040, Tx. 31 651 epo nl,
Fax: (+31-70) 340-3016

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Holtorf, S

INTERNATIONAL SEARCH REPORT

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PCT/US 97/00983

| C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT | | |
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| Category * | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
| X | WO 94 08031 A (CONSORTIUM ELEKTROCHEM IND :WICH GUENTER (DE); LEINFELDER WALFRED) 14 April 1994 see the whole document --- | 1 |
| X | PLANT MOLECULAR BIOLOGY, vol. 26, 1994, pages 1085-1101, XP002032321 SHEN, B.S., ET AL. : "PARTIAL SEQUENCING AND MAPPING OF CLONES FROM TWO MAIZE cDNA LIBRARIES" see the whole document --- | 6-8 |
| X | EMBL SEQUENCE DATA LIBRARY, 1 October 1994, HEIDELBERG, GERMANY, XP002032322 SHEN, B., ETAL. : "PARTIAL SEQUENCING AND MAPPING OF CLONES FROM MAIZE cDNA LIBRARIES" ACCESSION No.T25248 --- | 6-8 |
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INTERNATIONAL SEARCH REPORTPCT Application No
PCT/US 97/00983

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

| Category | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
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| A | PLANT PHYSIOLOGY, vol. 71, 1983, pages 136-140, XP000674466 RANCH, J.P., ET AL. : "EXPRESSION OF 5-METHYLTRYPTOPHAN RESISTANCE IN PLANTS REGENERATED FROM RESISTANT CELL LINES OF DATURA INNOXIA" see the whole document ----- | 1-37 |

PCT

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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

| (51) International Patent Classification ⁶ : C12N 15/53, 9/02, C12Q 1/68 | | A3 | (11) International Publication Number: WO 99/14336 (43) International Publication Date: 25 March 1999 (25.03.99) | | | | | | | | | | | | | | | | | | | | |
|--|-----------|--|--|------------|-----------|-------------|------------|---|-----|-----|-----|----|----|----|----|----|----|----|---|----|----|----|---|
| (21) International Application Number: PCT/US98/19494 (22) International Filing Date: 18 September 1998 (18.09.98) (30) Priority Data: 60/059,379 19 September 1997 (19.09.97) US | | (81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, GM, HR, HU, ID, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG). | | | | | | | | | | | | | | | | | | | | | |
| (71) Applicant (for all designated States except US): PROMEGA CORPORATION (US/US); 2800 Woods Hollow Road, Madison, WI 53711-5399 (US). (72) Inventors; and (75) Inventors/Applicants (for US only): WOOD, Keith, V. [US/US]; 902 Kottke Drive #5, Madison, WI 53719 (US). HALL, Mary, P. [US/US]; 6305 Dyllyn Drive, Madison, WI 53719 (US). (74) Agent: MARTIN, Alice, O.; Brinks Hofer Gilson & Lione, P.O. Box 10087, Chicago, IL 60610 (US). | | Published <i>With international search report. Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i> (83) Date of publication of the international search report: 3 June 1999 (03.06.99) | | | | | | | | | | | | | | | | | | | | | |
| (54) Title: THERMOSTABLE LUCIFERASES AND METHODS OF PRODUCTION | | | | | | | | | | | | | | | | | | | | | | | |
| <p>Stability at 37C normalized to t=0</p> <table border="1"><caption>Data for Stability at 37C normalized to t=0</caption><thead><tr><th>Time (min)</th><th>PPE-2 (%)</th><th>39-5B10 (%)</th><th>49-7C6 (%)</th></tr></thead><tbody><tr><td>0</td><td>100</td><td>100</td><td>100</td></tr><tr><td>10</td><td>95</td><td>95</td><td>10</td></tr><tr><td>20</td><td>90</td><td>90</td><td>0</td></tr><tr><td>60</td><td>90</td><td>90</td><td>0</td></tr></tbody></table> | | | | Time (min) | PPE-2 (%) | 39-5B10 (%) | 49-7C6 (%) | 0 | 100 | 100 | 100 | 10 | 95 | 95 | 10 | 20 | 90 | 90 | 0 | 60 | 90 | 90 | 0 |
| Time (min) | PPE-2 (%) | 39-5B10 (%) | 49-7C6 (%) | | | | | | | | | | | | | | | | | | | | |
| 0 | 100 | 100 | 100 | | | | | | | | | | | | | | | | | | | | |
| 10 | 95 | 95 | 10 | | | | | | | | | | | | | | | | | | | | |
| 20 | 90 | 90 | 0 | | | | | | | | | | | | | | | | | | | | |
| 60 | 90 | 90 | 0 | | | | | | | | | | | | | | | | | | | | |
| (57) Abstract | | | | | | | | | | | | | | | | | | | | | | | |
| <p>Luciferase enzymes with greatly increased thermostability, e.g., at least half lives of 2 hours at 50 °C, cDNAs encoding the novel luciferases, and hosts transformed to express the luciferases, are disclosed. Methods of producing the luciferases include recursive mutagenesis. The luciferases are used in conventional methods, some employing kits.</p> | | | | | | | | | | | | | | | | | | | | | | | |

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 97/00983

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. Claims Nos.: because they relate to subject matter not required to be searched by this Authority, namely:

2. Claims Nos.: because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
Remark: Claim 22 was read as referring to the method of claim 10.

3. Claims Nos.: because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

The additional search fees were accompanied by the applicant's protest.
 No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Patent Application No
PCT/US 97/00983

| Patent document cited in search report | Publication date | Patent family member(s) | Publication date |
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INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 98/19494A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/53 C12N9/02 C1201/68

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

| Category | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
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 Further documents are listed in the continuation of box C. Patent family members are listed in annex.

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INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 98/19494

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

| Category | Character or document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 98/19494

Box I Observations where certain claims were found unsearchable (Continuation of Item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. Claims Nos.: because they relate to subject matter not required to be searched by this Authority, namely:

2. Claims Nos.: 16, 21 because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
The claims 16, 21 and 22 could not be searched due to the fact that the sequences are not specified.

3. Claims Nos.: because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of Item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

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4. No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

The additional search fees were accompanied by the applicant's protest.

No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

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